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IL-1R8: a novel checkpoint regulating anti-tumor and anti-viral activity of NK cells

Thesis submitted by

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1. Abstract

IL-1R8 is an Interleukin-1 receptor family member that acts as a negative regulator of IL-1 family receptor and Toll-like receptor (TLR) signaling. Both murine and human NK cells express high levels of IL-1R8 but its functional role in this cell type has not been described so far. Natural Killer (NK) cells are innate lymphoid-derived cells and are able to recognize damaged, stressed, viral infected or tumor cells, which express ligands interacting with activating NK cell receptors. The expression of IL1R8 in NK cells prompted us to analyze its potential role in controlling NK cell effector functions.

Expression analysis showed that IL-1R8 was acquired during differentiation in human and murine NK cells. IL-1R8 deficiency in the mouse was associated with higher frequency and absolute number of mature NK cells in blood, spleen, bone marrow and liver. Moreover, IL-1R8 deficient NK cells display an increased Interferon γ (IFN γ), Granzyme B and Fas ligand expression and degranulation. IL-18, which is a key regulator of NK cell activities and can be targeted by IL-1R8, was responsible for this phenotype. Indeed, IL-1R8 regulated IL-18 axis during NK cell differentiation and activation and IL-18-dependent activation of mTOR and JNK pathways increased in IL-1R8-deficient NK cells.

To assess the role of IL-1R8 in NK cells in pathology, we used models of 3-methylcholanthrene (MCA)-induced lung metastasis, colon cancer-derived liver metastasis and diethylnitrosamine (DEN)-induced hepatocellular carcinoma. The number and dimension of liver and lung metastasis and the liver disease severity were significantly reduced in *Il1r8*^{-/-} mice. The depletion of NK cells in these models totally abrogated the protection observed in *Il1r8*^{-/-} mice. Finally, we investigated the role of IL-1R8 in NK cell antiviral activity, in a model of murine cytomegalovirus (MCMV) infection. *Il1r8*^{-/-} mice showed an improved virus control in the liver and the protection was associated with enhanced NK cell degranulation

and IFN γ production. The adoptive transfer of *Il1r8*^{-/-} NK cells conferred protection in both metastasis and viral infection models.

Collectively, these results showed that IL-1R8 played a non-redundant role in the regulation of NK cell development and effector functions by tuning IL-18-dependent activities. IL-1R8 therefore emerges as a novel checkpoint molecule modulating NK cell antitumoral and antiviral potential. Preclinical models showed that the inactivation of IL-1R8 unleashed NK cell effector functions, unveiling IL-1R8 as a potential immunotherapy target in the context of cancer and viral infections.

2. Introduction

2.1 Interleukin-1 receptor 8 (IL-1R8)

2.1.1 The IL-1 family

Interleukin-1 receptor family members (ILRs) and Toll Like Receptors (TLRs) belong to a superfamily characterized by a common intracellular signalling domain, named Toll-IL-1 resistance (TIR) domain [1], and extracellular Ig-like domains or leucine-rich repeats [2]. ILRs and TLRs are phylogenetically conserved proteins involved in the initiation and amplification of inflammation and innate and adaptive immune responses. TLRs work as sensors for exogenous infectious agents and host tissue injury, recognizing specific pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The ILR subfamily is composed by receptors and accessory proteins (AcP) for the cytokines of the IL-1 family. A novel nomenclature of ILRs has been recently proposed [3] and it is as follows: IL-1R1 (IL-1RI), IL-1R2 (IL-1RII), IL-1R3 (IL-1RAcP), IL-1R4 (ST2), IL-1R5 (IL-18R α), IL-1R6 (IL-1Rrp2, IL-36R), IL-1R7 (IL-18R β), IL-1R8 (also known as TIR8 or SIGIRR), IL-1R9 (TIGIRR-2), IL-1R10 (TIGIRR-1). The IL-1 system includes molecules with agonist activity (IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , and IL-36 γ), three receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37) (Figure 1).

ILR and TLR signaling covers a wide spectrum of functions in several tissues and cell types, ranging from tissue homeostasis regulation to protective responses against infections and modulation of inflammation. Given the huge capacity of ILR and TLR pathways to drive

inflammatory responses, the strict regulation of this system plays a significant role in both physiological and pathological conditions.

Upon ligand binding, TLRs as well as ILRs dimerize through their TIR domains, inducing the recruitment of TIR domain containing adapter proteins, in particular MyD88, MAL, TRIF, TRAM and SARM, which couple to downstream protein kinases (e.g. IL-1R associated kinases (IRAKs), and tumor necrosis factor receptor-associated factor 6 (TRAF6)). The signal leads to the activation of key transcription factors associated with inflammatory and immune responses, such as nuclear factor- κ B (NF κ B), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-associated protein kinase, extracellular signal-regulated kinases

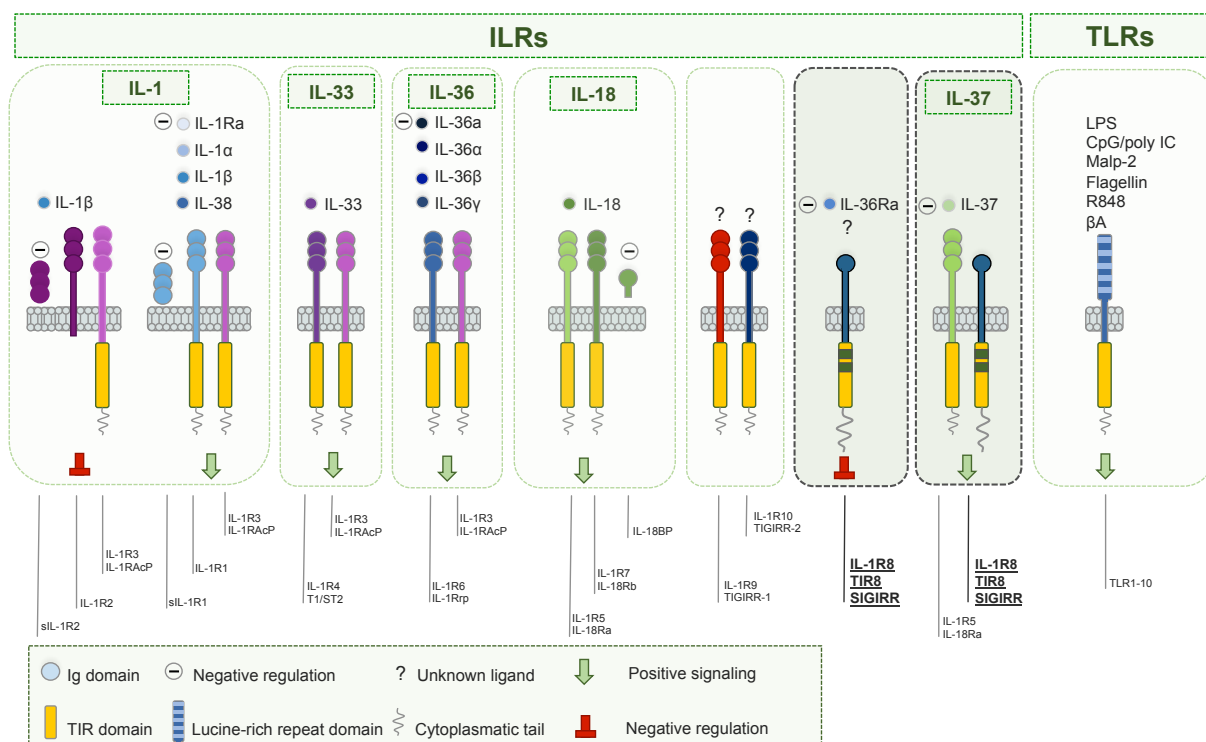


Figure 1. Members of the IL-1 and Toll-like receptor family.

IL-1 family members comprise receptors (IL-1R1, IL-1R4, IL-1R5, IL-1R6, IL-1R7, IL-1R9 and IL-1R10). The IL-1 system includes an accessory protein (IL-1R3), receptors that work as negative regulators (IL-1R8 and IL-1R2), molecules with agonist activity (IL-1 α , IL-1 β , IL-1 δ , IL-33, IL-36 α , IL-36 β , and IL-36 γ), three receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37). IL-1 and Toll-like receptors all share a conserved TIR domain, that shows two mutations in IL-1R8.

(ERKs), mitogen-activated protein kinases (MAPKs), and members of the interferon (IFN)-regulatory factor (IRF) [4-6].

ILR and TLR functional activation is modulated by several and diverse mechanisms. Among these, IL-1R2 exerts regulatory functions acting as decoy receptor for IL-1, dominant negative molecule and scavenger [7, 8]. In addition, it is also present in the cytoplasm where it binds pro-IL-1 α , preventing its cleavage and activation [9]. IL-1R8, also known as TIR8 or SIGIRR, whose function will be detailed, is a fringe member of the family that lacks conventional signalling capacities and works as a negative regulator of the family, acting intracellularly. Available information suggests that IL-1R8 interferes with the association of TIR-containing adaptor molecules to the receptor complex, thus dampening the signalling pathway leading to signal transduction [10, 11]. In addition, IL-1R8 is a component of the receptor recognizing the anti-inflammatory cytokine IL-37 [12]. IL-37 is an anti-inflammatory cytokine that acts as a natural brake of inflammation, signaling through IL-1R5/IL-18R α and IL-1R8 was recently described as a co-receptor, required for the formation of the tripartite complex IL-37-IL-1R5/IL-18R α -IL-1R8 [12]. IL-18BP is an extracellular protein that binds IL-18, preventing its interaction with the receptor IL-1R5/IL-18R, and thus neutralizing its activity [13-15]. IL-1Ra and IL-36Ra are receptor antagonists that bind IL-1R1 and IL-1R6, respectively [6, 16-19].

Intracellular negative regulators of ILRs and TLRs and specific miRNAs (miR-155, miR-21, miR-146a, miR-132, miR-9, and miR-147) targeting ILR and TLR signaling have been also reported [20-24]. The abundance of these regulatory mechanisms highlights the relevance of the negative regulation of both ILRs and TLRs, which if uncontrolled, may activate detrimental inflammation and cause tissue damage. For instance, local and systemic inflammation induced by IL-1 underlay a broad list of diseases, ranging from rheumatic diseases and autoinflammatory syndromes to cardiovascular diseases, type 2 diabetes and

infections and sepsis, and targeting of IL-1 has relevant therapeutic implications [25-28].

2.1.2 IL-1R8 (TIR8/SIGIRR)-mediated regulation of ILRs and TLRs

IL-1R8 was identified by our group and reported as TIR8 in 1998 (Accession number: AF113795), and in parallel by John Sims' group in 1999 and reported as SIGIRR (Figure 1) [29]. IL-1R8 is localized on human chromosome 11 and murine chromosome 7 and is composed by ten exons in humans and nine exons in the mouse. [29]. The human protein is 410 aminoacid-long and displays unique features compared with other ILRs. Indeed, it is composed of a single extracellular Ig domain, in contrast with the other family members, which have three, a transmembrane domain, a cytoplasmic TIR domain and an unusually long tail (95 residues), which is missing in other TIR domain-containing receptors. The IL-1R8 TIR domain lacks two conserved residues (Ser447 and Tyr536), which are replaced by Cys222 and Leu305 suggesting unconventional signaling [30, 31].

IL-1R8 sequence is highly conserved among vertebrates, from chicken to human in terms of sequence and pattern of expression. Human and murine protein sequences are 82% identical and share 23% overall identity with IL-1R1 [32].

IL-1R8 has 5 potential N-glycosylation sites in the extracellular domain in humans and 4 in the mouse and is extensively N- and O-glycosylated. It was recently showed that loss of N-linked glycosylation was associated with an inactive isoform of IL-1R8, generated by alternative splicing in colon cancer cells. Moreover, loss of complex glycan modifications was sufficient to suppress IL-1R8 activity *in vivo*, highlighting that post-transcriptional modifications are required for the functional activity of IL-1R8 [33].

IL-1R8 is widely expressed in several epithelial tissues, in particular by epithelial cells of the

Regulatory functions of IL-1R8

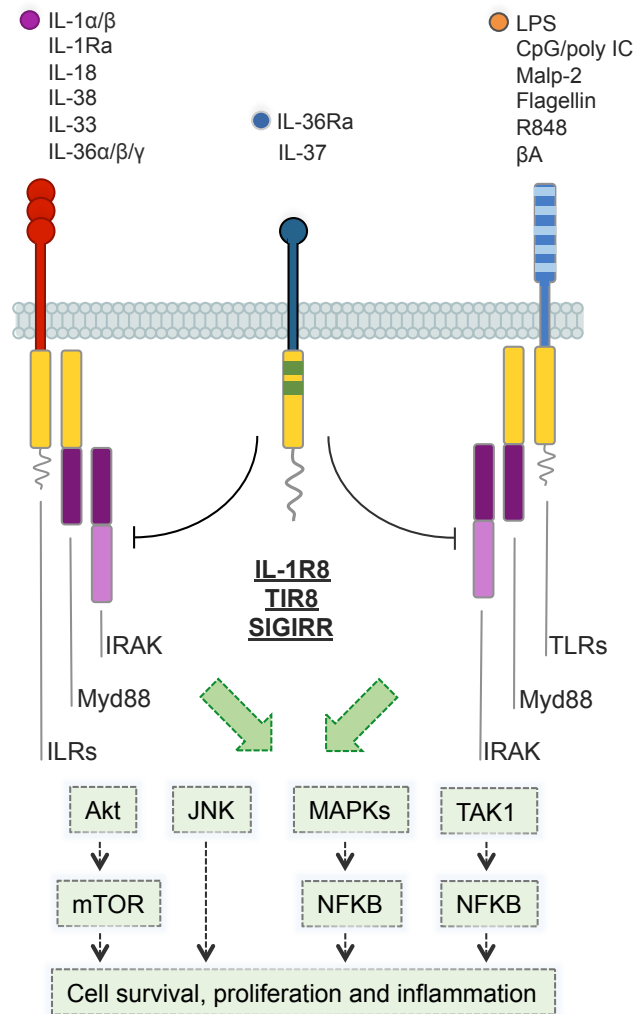


Figure 2. IL-1R8 regulatory function.

IL-1R8 negatively regulates IL-1 and Toll-like receptors and it is therefore involved in the modulation of cell survival, proliferation and inflammation. It exerts its inhibitory activity directly acting on cell-membrane receptors but also TIR-containing adaptor molecules (e.g. Myd88). It inhibits IL-1 and TLR downstream pathways such as MAPK and mTOR pathways. With a different mechanism, it is also necessary for the signaling of the anti-inflammatory cytokine IL-37, therefore acting both as a negative regulator and an active anti-inflammatory mediator.

kidney, digestive tract, liver, lung, and in lymphoid organs. Among leukocytes, it is expressed by monocytes, B and T lymphocytes, dendritic cells and NK cells [29, 34]. Little is known about the regulation of IL-1R8 expression and the stimuli and pathways involved. In general,

both IL-1R8 mRNA and protein expression are reduced in inflammatory conditions.

IL-1R8 exerts its regulatory activity by inhibiting NF κ B and JNK activation induced by TIR-containing ILRs or TLRs upon ligand binding, but not by other receptors such as Tumor necrosis factor (TNF) receptors. In particular, IL-1R8 was shown to modulate the activation of IL-1R1, IL-1R5/IL-18R α , IL-1R4/ST2, TLR4, TLR7, TLR9, TLR3 and TLR1/2 (Figure 2) [30, 31, 35-37].

The molecular mechanism involved in IL-1R8-mediated regulation of ILRs and TLRs is still poorly characterized. Upon stimulation with IL-1, the IL-1R8 extracellular domain was shown to block the dimerization between IL-1R1 and IL-1R3/IL-1RAcP, and the intracellular

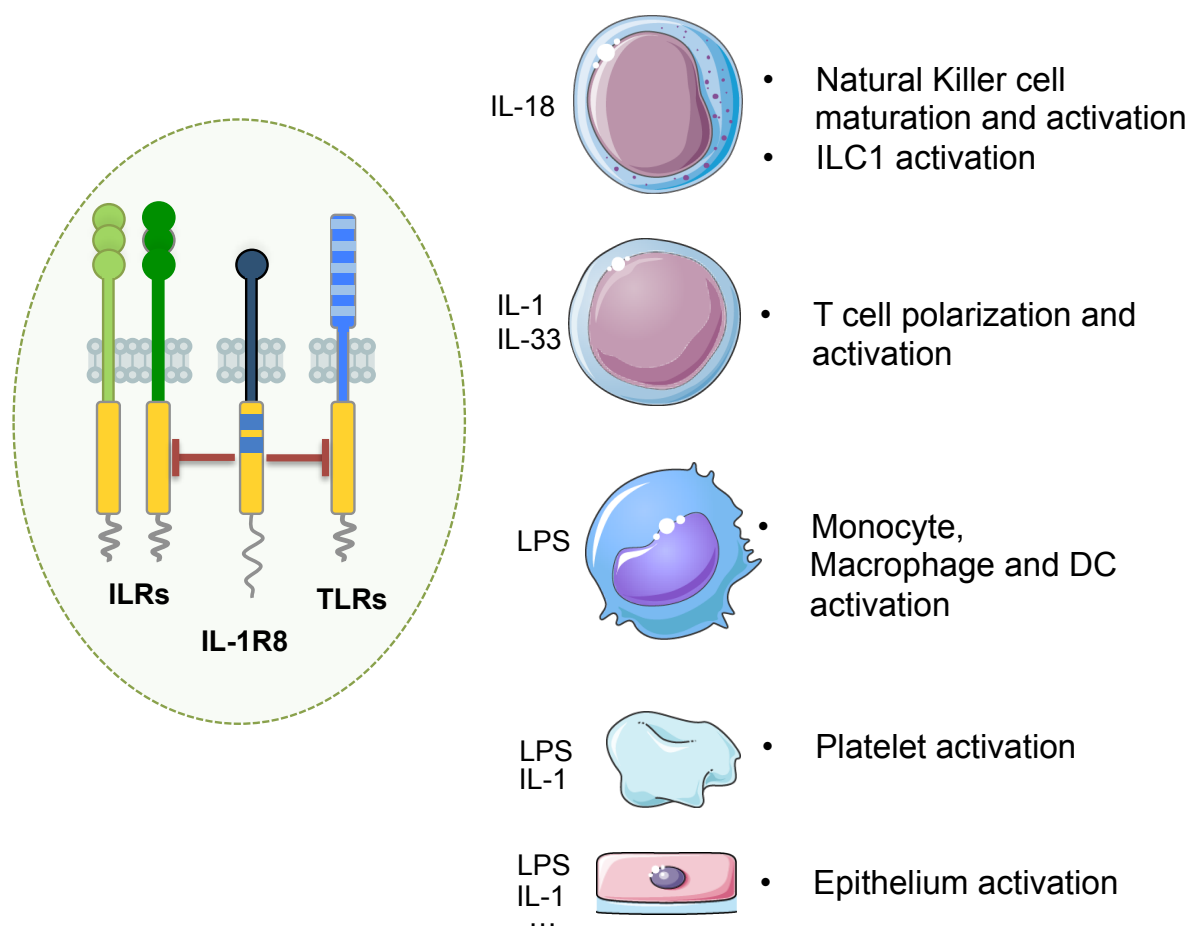


Figure 3. IL-1R8 activity in different cell types.

IL-1R8 is expressed by several cell types and exerts cell- and site-specific functions, which are also dependent on the context and involve diverse upstream mediators. It inhibits NK cell and ILC1 effector functions, it regulates T cell activation and polarization and it modulates myeloid cell, platelet and epithelial cell activation.

TIR domain was shown to bind the TIR-containing adaptor protein Myd88 and downstream signaling molecules (IRAK and TRAF6), thus modulating IL-1 signalling [31, 35]. Similarly, the targeting of IL-1R4/ST2 was shown to be dependent on both the extracellular immunoglobulin and TIR domains [37]. In contrast, only the TIR domain was necessary for the inhibition of TLR4 signalling, as demonstrated by mutagenesis studies [31, 35].

JNK and mTOR phosphorylation were shown to be enhanced in IL-1R8 deficient Th17 cells. IL-1R8 is therefore crucial in the modulation of metabolism, differentiation, expansion and effector functions of Th17 cells (Figure 2) [38]. IL-1R8 was also demonstrated to target mTOR phosphorylation driven by IL-1 or TLR agonists derived from commensal flora, in intestinal epithelial cells [39]. IL-1R8 therefore emerges as a regulator of the cell cycle, playing a crucial role in homeostatic conditions.

Thus, these results indicate that IL-1R8 interferes with the formation of TIR domain signalosome, preventing the dimerization of receptors, accessory proteins and adaptor molecules, and blocking TLR and IL-1 family signaling (Figure 2, 3).

IL-37 signaling

IL-1R8 was considered an orphan receptor, lacking a specific ligand. IL-37 has been recently demonstrated to bind IL-1R8, to generate the tripartite complex IL-37-IL-1R5/IL-18R α -IL-1R8 [12]. IL-37 is an anti-inflammatory cytokine that dampens the inflammatory response triggered by TLRs and cytokines in peripheral blood mononuclear cells (PBMCs), in macrophages and epithelial cells and IL-37-transgenic mice (IL-37tg mice) are protected in different inflammatory pathological conditions [40]. Recently, advanced imaging analysis

revealed a rapid interaction of IL-37 with both IL-1R5/IL-18R α and IL-1R8 in human PBMCs and bone marrow-derived macrophages (BMDMs) of IL-37tg mice upon stimulation with lipopolysaccharide (LPS) [12]. IL-1R8 and IL-1R5/IL-18R α were both required to support the anti-inflammatory activity of IL-37 in PBMCs, THP-1 macrophages and A549 epithelial cells. Finally, proteomic and transcriptomic analysis demonstrated that the IL-37-IL-1R5/IL-18R α -IL-1R8 complex triggered multiple signalling events leading to anti-inflammatory responses, such as inhibition of MAPK, NF κ B, mTOR, TAK1 and Fyn and activation of STAT3, Mer, PTEN and p62(dok).

Thus, IL-1R8 acts as a co-receptor for IL-1R5/IL-18R α upon IL-37 binding and it is required for the anti-inflammatory activity of IL-37 [12, 41]. This mechanism is relevant *in vivo*, since IL-1R8-deficiency abolished the protection of IL-37tg mice against endotoxin challenge or the protective role of IL-37 in a model of non-resolving *A. fumigatus* infection and pulmonary damage [12, 42]. Moreover, in a model of OVA-induced asthma, IL-37-driven anti-inflammatory effects were abolished in mice lacking either IL-1R5/IL-18R α or IL-1R8 [43].

Thus, these results demonstrate that IL-1R8 is not only a negative regulator, but also part of the receptor complex of the anti-inflammatory cytokine IL-37 and mediates an anti-inflammatory signaling activation (Figure 3).

2.1.3 Role of IL-1R8 in infections

IL-1R8 emerged as a key molecule in bacterial and fungal infections, playing a crucial role in the regulation of pathogen-induced TLR and ILR responses and in the modulation of inflammation and tissue damage (Figure 4).

In *Mycobacterium tuberculosis* infection, IL-1R8-deficient mice exhibited higher mortality, which was driven by an exacerbated systemic inflammatory response, even if no difference in

tissue bacterial load in the lung, liver or spleen was observed. Indeed, IL-1R8 deficient mice presented an overwhelming inflammatory response, characterized by enhanced macrophage and neutrophil lung infiltration and increased systemic levels of inflammatory cytokines. [44]. In a model of keratitis induced by *P. aeruginosa*, IL-1R8 was involved in the regulation of IL-1R1 and TLR4 signalling in T cells, and dampening Th1 response, thus preventing tissue damage and promoting resistance to infection [45]. Similarly, in acute lung infections caused by *P. aeruginosa*, IL-1R8-deficient mice showed increased susceptibility to the pathogen, in terms of mortality and bacterial load, increased production of pro-inflammatory cytokines and the phenotype was dependent on IL-1 signaling [46].

In *Candida albicans* or *Aspergillus fumigatus* infections, the absence of IL-1R8 led to increased susceptibility to mucosal and disseminated or lung infections, respectively [47]. IL-1R8-deficient mice showed increased mortality and fungal burden, enhanced activation of IL-1 signalling, Th17 and $\gamma\delta$ T cell response and reduced Treg activation. [47].

The role of IL-1R8 is therefore attributable to the negative regulation of IL-1 signalling in *P. aeruginosa*, *C. albicans* and *M. tuberculosis* infections, since IL-1 neutralization was sufficient to abolish the phenotype in IL-1R8-deficient mice.

The relevance of these data in the mouse was supported by a case-population study design in Vietnam, showing that 3 SNPs (rs10902158, rs7105848, rs7111432) in IL-1R8 gene correlated with the development of both pulmonary tuberculosis and tuberculous meningitis. Moreover, coinheritance of these SNPs with previously identified polymorphisms in TLR2 and TIRAP was associated with enhanced risk of susceptibility [48].

The protective role of IL-1R8 in the infections mentioned above is due to the regulation of ILR and TLR signalling that potentially cause detrimental inflammation and tissue damage. However, in a model of experimental urinary tract infection (UTI) induced by uropathogenic *Escherichia coli*, IL-1R8-deficient mice displayed reduced renal bacteria

outgrowth and diminished renal dysfunction [49].

Similarly, during pneumonia and sepsis induced by *Streptococcus pneumoniae* in the mouse, IL-1R8-deficiency was associated with delayed mortality, reduced bacterial load in the lungs and reduced dissemination of the infection [50]. Increased interstitial and perivascular inflammation was observed in IL-1R8-deficient lungs mice in the early phase of infection. Thus, IL-1R8 suppressed the protective antibacterial immune response in *S. pneumoniae* induced pneumonia [50].

Murine models of *Citrobacter rodentium* infection resemble human intestinal infections driven by enteric bacterial pathogens such as enterohemorrhagic *E. coli* (EHEC) and *Salmonella typhimurium*. Upon *Citrobacter rodentium* infection, IL-1R8-deficiency correlated with accelerated intestinal epithelial cell (IEC) proliferation and enhanced pro-inflammatory and anti-microbial response. However, IL-1R8 was shown to be protective in this model, in terms of weight loss, intestinal damage, colitis score and intestinal bacterial burden. IL-1R8 protective function was dependent on IL-1R1-MyD88 signalling, indicating that IL-1R1 may be the key receptor targeted by IL-1R8 in this context. Infected IL-1R8-deficient mice underwent a more rapid and dramatic loss of commensal flora, compared with controls. In infected mice, the microbiota depletion was directly dependent on the exacerbated antimicrobial response occurring in IL-1R8-deficient mice that favored pathogen colonization. Thus, IL-1R8-mediated regulation of IECs is responsible for the inhibition of a strong anti-microbial response that would otherwise lead to a rapid depletion of the commensal microbiota, during intestinal infection. In turn, the absence of competing microflora would favor the colonization by bacterial pathogens [51].

Recently, it was observed that IL-1R8 is highly expressed in both human and murine platelets and megakaryocytes and plays a key role in the regulation of platelet activation in inflammation and thromboembolism [52]. In particular, IL-1R8-deficient platelets displayed a

hyperactivation in basal conditions, in terms of active $\alpha 2b\beta 3$ and P-selectin surface expression. IL-1R8-deficiency was also associated to increased platelet/neutrophil aggregate formation, induced by LPS, IL-1 β or IL-18 *in vitro* and in a systemic LPS-induced inflammation model *in vivo* [52]. Hyperactivity of platelets in absence of IL-1R8 was demonstrated to be dependent on IL-1 signalling, since the phenotype was abrogated in *Il1r8*^{-/-}/*Il1r1*^{-/-} mice. Importantly, in patients with SIRS/sepsis, which is associated to platelet dysfunction [53], IL-1R8 surface expression was significantly down regulated compared to healthy controls and its expression level was associated with the severity of the disease. In line with this, murine platelets and megakaryocytes showed a reduced expression of IL-1R8 upon treatment with LPS *in vitro* and *in vivo*. LPS induced microparticle (MP) release from platelets and it was observed that MP derived from LPS-stimulated platelets or from serum of septic patients expressed higher levels of IL-1R8 compared to controls, suggesting a shedding of the receptor in inflammatory conditions through MP release [52]. These data elucidate a novel function of IL-1R8, characterizing its non-redundant role in the regulation of thrombocyte function.

Thus, IL-1R8 plays a crucial role in favoring the maintenance of a delicate equilibrium between the protective immune response against infections and the development of detrimental inflammation and host injury. The activity of IL-1R8 is therefore strictly dependent on the context and several lines of evidence suggest that during homeostasis the constitutive expression of IL-1R8 protects against inappropriate responses, whereas its down-regulation during acute inflammatory stimulation enhances the efficacy but also pathogenic potential of antibacterial host defense.

2.1.4 role of IL-1R8 in the CNS

IL-1R8 is expressed in the brain by neurons, microglia and astrocytes and it was shown to be involved in the regulation of LPS responsiveness in the brain (Figure 4) [17, 34, 54]. Indeed, IL-1R8-deficiency was associated with a massive LPS-induced inflammation in the brain. In response to LPS, IL-1R8 negatively regulated CD40, ICAM and cytokine (IL-6 and TNF α) mRNA expression in microglial cells and cytokine production in hippocampal tissue [55].

In addition, it has been observed that cognitive and synaptic functions, such as novel object recognition, spatial reference memory, and long-term potentiation (LTP) were impaired in IL-1R8-deficient mice, in absence of any external stimulus. This was associated with a higher expression of IL-1R1 and TLR4 and an enhanced activation of downstream signalling molecules (IRAK1, c-Jun, JNK and NF κ B) [56]. IL-1 α and high mobility group box 1 (HMGB1), which activate IL-1R1 and TLR4, respectively, were proposed to play a central role in the phenotype observed [56]. Moreover, IL-1R6 antagonist (IL-36Ra) inhibits the IL-1- and LPS-induced inflammatory response in glial cells and this effect was absent in mixed glia prepared from IL-1R8-deficient mice, suggesting the involvement of IL-1R8 in the anti-inflammatory activity of IL-36Ra, possibly mediated through the production of IL-4 [17]. Finally, a recent study showed that IL-1R8 acted as a negative regulator of β -amyloid (A β) peptide-induced TLR2 signalling in the brain. A β is the main component of neuritic plaques in Alzheimer's disease (AD) and the primary mediator of the AD-associated neuroinflammation [57]. Anti-TLR2 treatment of microglia attenuated the inflammatory response and the impairment in LTP, both induced by A β , confirming the central role of TLR2 in the A β -induced neuroinflammation. [57]. These findings highlighted the key role of IL-1R8 in the modulation of TLR2-induced inflammation in the brain and its relevance in a potential therapeutic approach targeting TLR2 in AD-related pathology.

A recent study elucidated the molecular mechanisms underlying cognitive and synaptic function impairment in absence of IL-1R8 [58]. IL-1R8-deficiency affected neuron synapse morphology, plasticity and function, through the hyperactivation of IL-1R1 signaling. Indeed, IL-1R8-deficient hippocampal neurons exhibited a defective synaptic plasticity and function, in terms of an increased number of immature, thin spines and a decreased number of mature, mushroom spines along with a significant reduction of spine width, and reduced amplitude of miniature excitatory postsynaptic currents. The unleashed IL-1R1 activation in absence of IL-1R8 was shown to impair spine morphogenesis and plasticity through the PI3K/AKT/mTOR pathway by increasing the expression of methyl-CpG-binding protein 2 (MeCP2), a synaptopathy protein involved in neurological diseases characterized by defective plasticity, impaired cognition and intellectual disability, such as Rett syndrome and MeCP2 duplication syndrome [59]. IL-1 pharmacological inhibition with IL-1Ra (Anakinra) or IL-1R1 genetic inactivation rescued MeCP2 expression and cognitive deficit, suggesting that the physiological activation level of IL-1 signaling is fundamental for the proper neural long-term potentiation [58]. Interestingly in this context, IL-1 inhibition in cryopyrin-associated periodic syndrome (CAPS) patients, not only reduced signs and symptoms of IL-1-dependent inflammation, but also reversed mental defects [60]. These results thus identify IL-1R8 as a novel molecular player involved in synaptopathies acting by fine tuning IL-1 activity in neurons.

2.1.5 Role of IL-1R8 in sterile inflammation

IL-1R8 in autoimmunity

ILRs and TLRs are key players in the pathogenic mechanisms of autoimmune disorders and IL-1R8 emerged as a crucial regulator in this context (Figure 4). In particular, IL-1 regulates

the differentiation and function of Th17 cells, which are involved in inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis, psoriasis, and inflammatory bowel disease (IBD) [61]. IL-33 is a driver of type 2 inflammatory responses and is implicated in allergy and asthma [62].

Gulen et al. showed that IL-1R8 was induced during Th17 cell polarization and that it controlled Th17 cell differentiation, expansion and effector functions, through the direct inhibition of IL-1 signalling in T cells. An increased phosphorylation rate of JNK, mTOR and 4EBP1 was observed in IL-1R8-deficient T cells, upon stimulation with IL-1. In particular, IL-1-induced mTOR pathway was critical for the IL-1R8-mediated modulation of Th17 response. Thus, IL-1R8 emerged as a key regulator of IL-1 activity in Th17 cells and it was also observed to be involved in the Th17-mediated development of central nervous system (CNS) autoimmune disorders. Indeed, IL-1R8-deficient mice revealed higher susceptibility to experimental autoimmune encephalomyelitis (EAE), due to an increased Th17 infiltrate in the CNS and enhanced Th17 polarization and pathogenic functions [38].

In rheumatoid arthritis models, IL-1R8-deficient mice were shown to develop a more severe disease, and this correlated with an increased cellular infiltration in the affected joints. IL-1Ra treatment reduced the susceptibility of IL-1R8-deficient mice in zymosan-induced arthritis, suggesting that IL-1 played a central role in this model. Moreover, IL-1R8 was shown to suppress the spontaneous release of cytokines in human RA synovial cells *in vitro*. [63]. In agreement with this study, IL-1R8 expression was reduced in peripheral blood of patients with psoriatic arthritis, compared with healthy donors [64]. Moreover, IL-1R8 deficiency caused enhanced susceptibility to psoriasis, associated to increased infiltration and activation of $\gamma\delta$ T cells, in both Aldara- and rIL-23-induced psoriasis models. Interestingly, IL-1R8 directly modulated IL-1-driven IL-17A expression by $\gamma\delta$ T cells *in vitro* and *in vivo* and IL-17A depletion abolished the phenotype observed in IL-1R8-deficient mice [65].

Increasing evidences implicated IL-1R8 in the pathogenesis of systemic lupus erythematosus. Indeed, altered TLR signalling in DCs and B cells is one of the driving mechanisms of this autoimmune disorder. In particular, immune complexes containing the lupus autoantigen U1snRNP or nucleosomes activate DCs and autoreactive B-cells via TLR7 and TLR9, respectively [66, 67]. In the mouse IL-1R8-deficiency alone did not induce autoimmunity against DNA. However IL-1R8-deficiency in C57BL/6^{lpr/lpr} mice, which develop delayed autoimmunity due to impaired Fas-induced apoptosis of autoreactive B and T cells, caused increased activation of DCs and B cells and production of proinflammatory cytokines and B cell antiapoptotic mediators. IL-1R8-deficient C57BL/6^{lpr/lpr} mice also displayed and increased production of autoantibodies (anti-dsDNAIgG, anti-nucleosome, anti-Sm antigen, anti-snRNP, and rheumatoid factor) and presented a massive lymphoproliferative disorder, associated with enhanced autoimmune lung disease, lupus nephritis and hypergammaglobulinemia, compared with IL-1R8-competent C57BL/6^{lpr/lpr} controls [68]. In line with this, IL-1R8 was also protective in a model of hydrocarbon oil-induced lupus, in which it modulated TLR7-mediated activation of DCs and expansion of autoreactive lymphocyte clones. IL-1R8 is therefore involved in the regulation of DC and B cell activation, by preventing exacerbated autoimmune reactions, lymphoproliferation and tissue damage in SLE [69]. The data in the mouse were supported by recent analysis of IL-1R8 involvement in SLE in human. A case study of a cohort of SLE patients revealed a reduced frequency of IL-1R8⁺ CD4⁺ T cells in the peripheral blood of SLE patients compared with healthy individuals. Moreover, the frequency of IL-1R8⁺ CD4⁺ T cells was further reduced in SLE patients with nephritis, compared with those without nephritis [70]. Moreover, a genetic variant of IL-1R8 (rs7396562) was identified and it was demonstrated to correlate with the susceptibility to SLE, in a Chinese population [71].

IL-1R8-mediated regulation in asthma and allergy

IL-33 signalling is a key driver of type 2 immunity, which favors protective immune responses in parasite infections and tissue repair but is also involved in pathological conditions such as asthma, allergy and eosinophilia (Figure 4) [72]. IL-33 receptor (IL-1R4/ST2) affects innate and adaptive lymphoid cells (ILCs and Th2), inducing the production of type 2 cytokines (IL-4, IL-5, IL-13), and can be targeted by IL-1R8. Indeed, IL-1R8 inhibits IL-33-mediated signalling in Th2 cells, controlling the production of type 2 cytokines *in vitro* and *in vivo* [37]. IL-1R8-deficient mice were shown to be hyper responsive to IL-33, in terms of lung inflammation, splenomegaly and increased serum levels of IL-5 and IL-13 [37]. Moreover, in a model of allergic pulmonary inflammation induced by OVA, IL-1R8 deficiency was associated with increased leukocyte lung infiltration, IL-5 and IL-4 levels and OVA-specific IgE induction, due to an exacerbated Th2 response [37]. These results indicate that IL-1R8 serves as a negative feedback control in Th2-polarization and restimulation, thus controlling allergic inflammatory responses. However, a genetic study performed on a cohort of Japanese asthma patients revealed that none of the alleles or haplotypes of IL-1R8 identified were associated with asthma susceptibility or asthma-related conditions [73].

Role of IL-1R8 in kidney sterile inflammation

IL-1R8 is expressed at high levels in the kidney, in particular by tubular epithelial cells. Immunohistochemical analysis revealed extensive IL-1R8 positivity in the majority of tubular epithelial cells of the renal cortex, showing a predominant expression at the apical side of renal proximal tubules (Figure 4) [36]. IL-1R8 was shown to be a key player in sterile kidney diseases, by regulating TLR activation by nucleosomes and DAMPs, released during ischemic cell necrosis and associated with pathological conditions such as lupus nephritis, postischemic acute renal failure or kidney transplantation [68, 69, 74, 75].

In a postischemic renal failure model, IL-1R8 deficiency was associated with increased renal injury, due to a massive activation of myeloid cells, increased intrarenal cytokine and chemokine production and increased leukocyte recruitment. [74]. In line with this, in a mouse model of fully mismatched kidney allotransplantation, IL-1R8-deficient grafts were less tolerated compared with control grafts, leading to acute rejection. Moreover, IL-1R8-deficiency was associated with an enhanced ILR- and TLR-driven post transplant kidney inflammatory response, in particular due to increased neutrophil and macrophage infiltrate and higher expression of TNF α and chemokines. The enhanced expansion and maturation of DCs observed in IL-1R8-deficient mice led to an amplified adaptive alloreactivity and reduced Treg cell development. Thus, IL-1R8 plays a key role in the regulation of the allogeneic immune response *in situ* and is involved in graft survival [75].

Role of IL-1R8 in intestinal inflammation

IL-1R8 was demonstrated to be a key regulator of intestinal homeostasis. Intestinal epithelial cells (IECs) are intrinsically hypo-responsive to bacterial products, thus preventing exaggerate inflammatory responses against the commensal flora but also limiting the enteric host defense (Figure 4) [30]. On the other hand, gut microflora-mediated activation of ILRs and TLRs provides the survival signals for IECs and this pathway is targeted by IL-1R8, which is therefore involved in controlling proliferation and survival in colon crypts [76]. The specific role of IL-1R8 in the regulation of microflora and microflora-derived signaling in the intestinal barrier is quite controversial, probably because of the animal-house-dependent variations in the microflora, and needs to be further characterized.

In a model of sterile colitis, induced by dextran sodium sulfate (DSS), IL-1R8-deficiency was associated with an exacerbated intestinal inflammation, in terms of weight loss, intestinal bleeding, local tissue damage and a reduced survival. This correlates with an

increased leukocyte infiltration in the intestine and higher level of proinflammatory cytokines (TNF α , IL-6, IL-1 β , IL-12p40, IL-17), chemokines (CXCL1, CCL2) and prostaglandins. Experiments with bone marrow chimeric mice demonstrated that the regulatory function exerted by IL-1R8 occurs in epithelial cells, in both DSS- and enteric pathogen-induced colitis [30, 76].

2.1.6 Role of IL-1R8 in cancer

Epidemiological studies have shown that chronic inflammation, both dependent on infectious agents or not, can increase the risk of cancer (Figure 4). The hallmarks of cancer-related inflammation are comparable to those observed in chronic inflammatory conditions: inflammatory cells and mediators are present in the tumor tissue and they are implicated in tissue repair, remodelling and angiogenesis. This 'smouldering inflammation' occurs even in tumors that are not directly caused by an inflammatory trigger. Cancer-related inflammation depends on two possible pathways: an intrinsic pathway, driven by oncogenic mutations that cause both neoplasia and inflammation, or an extrinsic pathway, driven by inflammatory conditions that favor tumor development (e.g. colitis-associated intestinal cancer) [77-80]. Several studies have revealed a crucial role of ILR and TLR signalling in this context, in which NF κ B is one of the key orchestrators, and that IL-1R8 plays a protective role in the pathogenesis of cancer-related inflammation in different murine models of colon cancer [80].

Colorectal cancer

IL-1R8 function in cancer was first characterized in a model of colorectal cancer, induced by the treatment with the procarcinogen Azoxymethane (AOM), followed by Dextran sodium sulfate (DSS), which favors chronic inflammation [76, 81]. This model mimics intestinal

cancer that develops in chronic IBD patients, in particular Ulcerative Colitis patients. In the AOM-DSS colitis-associated cancer (CAC) model, IL-1R8-deficiency was associated with exacerbated inflammation in the intestine, leading to increased susceptibility to cancer development, in terms of number, size, and severity of lesions. IL-1R8 negatively regulated intestinal permeability, *in situ* production of proinflammatory cytokines and chemokines and prostaglandin E₂, and the expression of NFκB-induced genes involved in cell survival and proliferation (Bcl-xL and Cyclin D1) [76, 81]. In this context, chemokines favored cancer progression, influencing the extent and type of leukocyte infiltrate (e.g. recruiting Th2 and Treg cells) and driving tumor cell and endothelial cell growth and migration [82, 83]. Moreover, increased levels of IL-10 and TGF-β were observed in tumors of IL-1R8-deficient mice, reflecting an immunosuppressive microenvironment that inhibited T cell-dependent antitumoral immunity [83]. IL-1R8 overexpression in gut epithelial cells abolished the susceptibility of IL-1R8-deficient mice to CAC development, suggesting that the regulatory activity of IL-1R8 in intestinal epithelial cells plays a central role in this model [76]. Since commensal microflora-derived stimuli are necessary for the homeostasis of colon epithelium and are involved in colitis-associated carcinogenesis, IL-1R8 regulation may be possibly dependent on its direct modulation of microbiota-activated TLRs [84]. However, IL-1R8-mediated targeting of other TLR- and ILR-related pathways cannot be excluded in this model.

IL-1R8 involvement in colon cancer was also investigated in the genetic Apc^{min/+} model, in which tumor initiation is caused by loss of heterozygosity (LOH) of the tumor suppressor Apc and which mimics the Familial Adenomatous Polyposis syndrome [85]. In Apc^{min/+} mice, IL-1R8 deficiency led to an increased susceptibility to cancer development, due to a more sustained activation of the Akt/mTOR pathway, which plays a crucial role in tumor initiation [86]. In this model, IL-1R8 was demonstrated to exert an antitumoral activity by suppressing IL-1- and TLR-induced mTOR-mediated cell cycle progression and

consequent genetic instability [39].

A recent study has investigated the role of IL-1R8 in human colorectal cancer, demonstrating that colon tumors express lower level of IL-1R8 compared with healthy tissues and that IL-1R8 is frequently inactivated in human colorectal cancer [33]. Indeed, Zhao et al. identified a dominant negative isoform of IL-1R8 (IL-1R8^{ΔE8}) and RNA sequencing data demonstrated that the expression level of this isoform increased in human colon cancer, compared with healthy tissue. The IL-1R8^{ΔE8} isoform originated from a transcript that lacks the exon 8 of the gene and exhibited compromised integrity of the TIR domain, increased retention in the cytoplasm and reduced N-linked glycosylation. The cytoplasmic retention caused a decrease in the cell surface expression of IL-1R8 and a consequent loss of its inhibitory activity. Moreover, IL-1R8^{ΔE8} isoform was shown to be able to interact with full-length IL-1R8, acting as an antagonist of IL-1R8 and thus suppressing its function. To investigate the mechanism responsible for IL-1R8^{ΔE8} isoform synthesis in tumor cells, sequence analysis were performed and predicted that exon 8 would be intrinsically a “weak” exon, with high probability of exclusion. Exon 8 also displayed a binding site for CTCF, a factor that favors the inclusion of weak exons, and since the binding can be reduced by methylation, hypermethylation was proposed to be the strategy followed by cancer cells that leads to IL-1R8^{ΔE8} isoform expression. Indeed, treatment with decitabine, a methyltransferase inhibitor, reduced IL-1R8^{ΔE8} isoform expression. To model the impact of IL-1R8^{ΔE8} isoform in colon carcinogenesis in the mouse, gut epithelium-specific IL-1R8 transgenic mice were generated, expressing a mutant form of IL-1R8 (IL-1R8^{N85/101S}) that mimics IL-1R8^{ΔE8} isoform or wild-type IL-1R8 as a control. In both AOM and AOM-DSS models, the presence of wild-type IL-1R8 in intestinal epithelial cells protected the mice from the development of colon cancer. On the contrary, mice expressing IL-1R8^{N85/101S} isoform had the same phenotype as IL-1R8-deficient mice, suggesting that complex glycan modifications and cell

surface expression are necessary for IL-1R8 functional activity *in vivo* [33]. Thus, IL-1R8 alternative splicing is an escape mechanism adopted by tumor cells to inactivate IL-1R8 through the generation of a dominant negative isoform.

Chronic lymphocytic leukemia

Both genetic defects and microenvironment stimuli contribute to chronic lymphocytic leukemia (CLL) development and progression. Moreover, factors originating from the microenvironment are involved in the selection and expansion of the malignant clone [87, 88]. Human malignant B cells expressed lower levels of IL-1R8 mRNA than normal B cells, and accordingly, in the well-established transgenic mouse model of CLL (TCL1), CD19⁺ B cells expressed lower levels of IL-1R8 mRNA transcript, compared with controls [88-91]. IL-1R8-deficiency did not affect B cell compartment in healthy mice, whereas it correlated with an earlier and more severe appearance of monoclonal B cell expansion and a reduced mouse life span in TCL1 transgenic mice, mimicking the aggressive variant of human CLL [91]. These findings revealed IL-1R8 inhibitory role in CLL initiation and progression, even though the molecular mechanism is still unclear. Endogenous TLR or ILR ligands are known to be involved in CLL and may be candidate targets of IL-1R8.

Breast cancer

Recently, Campesato LF et al. identified IL-1R8 in breast cancer as a crucial immunomodulatory molecule, which upregulation in transformed breast epithelial cells leads to an impaired innate immune sensing and T cell response [92]. Gene transfer experiments in breast tumor cell lines have shown that IL-1R8 upregulation in transformed cells inhibits IL-1-dependent NF- κ B activation and expression of pro-inflammatory molecules. IL-1R8-deficient mice were protected from the development of breast cancer in a genetic model

(MMTV-neu) and the number of lung metastasis was reduced. Experiments with bone marrow chimeric mice supported the predominant protective role of IL-1R8-deficiency in non-haematopoietic/tumor cells in this model. In particular *in vitro* and *in vivo* evidences demonstrated that IL-1R8 in tumor cells was responsible for shaping the tumor microenvironment and IL-1R8-deficiency was associated with higher frequency of DCs, NK cells and CD8⁺ T cells and lower proportions of TAMs [92]. In line with this, RNAseq analysis in 1102 clinical samples of breast tumors revealed that high IL-1R8 expression was associated with a non-T cell inflamed molecular signature, lower expression level of pro-inflammatory cytokines and chemokines, DC and NK cell metagenes, components of the peptide-presenting machinery, cytolytic enzymes and type I IFN-induced genes. Collectively, these data indicate that IL-1R8 expression in breast tumors represent a novel tumor-mediated immunoevasion mechanism, affecting the mobilization and activation of immune cells and therefore tumor growth and metastatization [92]. These findings have important therapeutic implication and the blockade of IL-1R8 in breast tumor cells may represent a way to restore the innate immune response and T cell trafficking and activation in the tumor microenvironment.

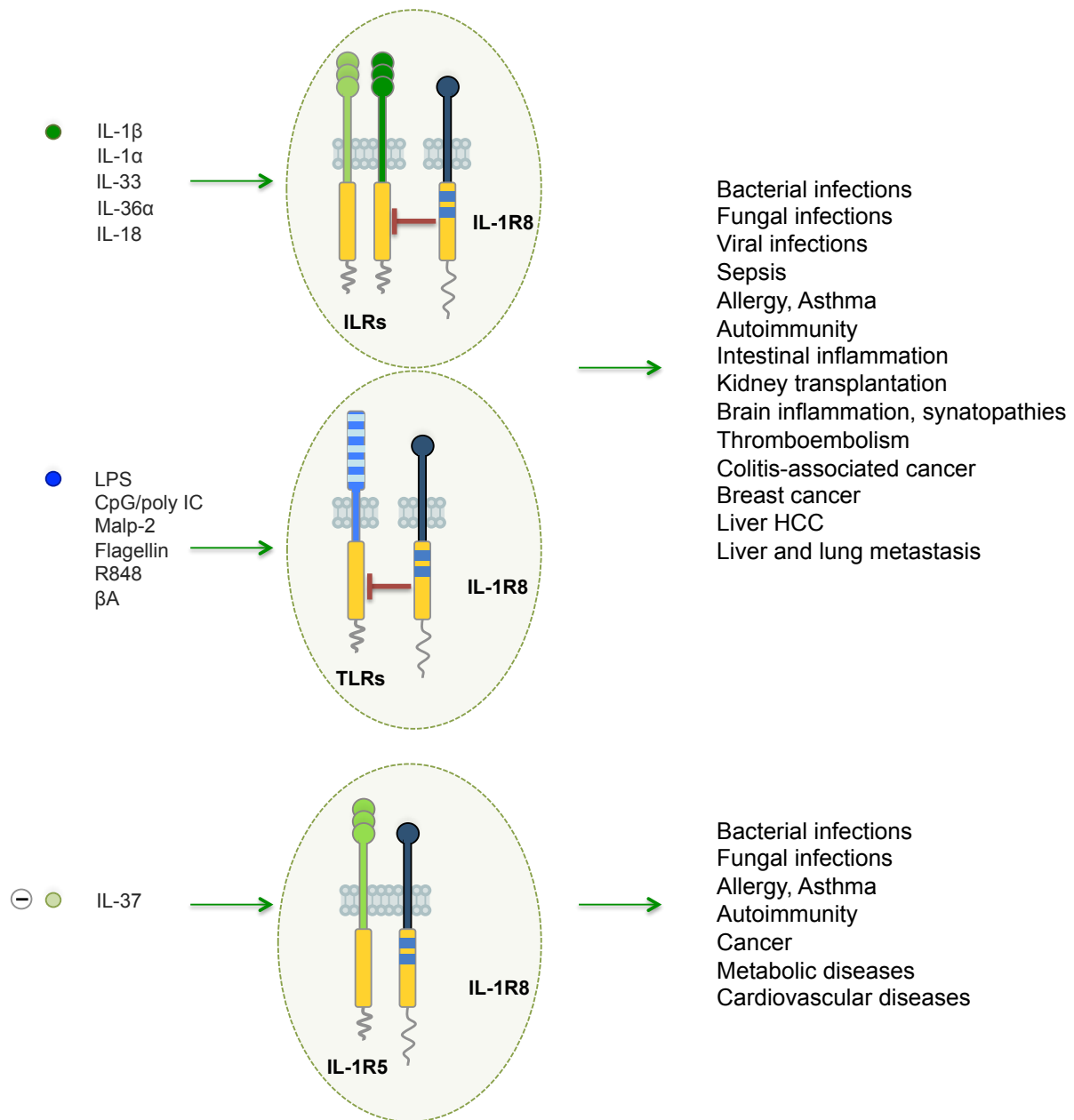


Figure 4. Role of IL-1R8 in different contexts.

IL-1R8-deficient mice showed that IL-1R8 is a crucial molecule regulating acute and chronic inflammation in several pathological conditions (listed in the figure). It is also involved in IL-37 signaling, being therefore relevant in different inflammatory contexts (listed in the figure).

2.2 Natural Killer cells

2.2.1 NK cell definition: a historical perspective

Natural Killer (NK) cells were originally described in 1975 independently by Kiessling and Herberman as lymphoid cells, “spontaneously” able to recognize and kill cancer and viral infected cells [93-96]. The first observation revealing the existence of this immune population was attempted in 1971, when Thornthwaite J.T. observed a particular murine cell type, first named CIPFC (Complement Independent Plaque Forming Cells) that was able to release a lytic agent and destroy the surrounding Sheep Red Blood Cells (SRBC), without any prior immunization [97, 98]. In 1975, these cells were called Natural Killer Cells and their capacity to destroy cancer cells was discovered. In particular, it was observed that the incubation of NK cells with a mastocytoma cell line (P815) caused the death of the tumor cells, occurring within minutes. They were defined as a “population of naturally occurring killer lymphocytes with specificity for tumor cells”. The term “natural” was used to underlie the spontaneous capacity of these cells to destroy target cells, differently from T cells, whose killing mechanism is antigen specific and MHC-restricted [99].

The acquisition of cytotoxic activity has been evolutionarily associated with a robust and tight regulation to control the cytolytic process and reduce tissue damage. In this regard, the NK cell activation mechanism was deeply investigated over the last twenty-five years and it was shown to be dependent on the concept of a dynamic equilibrium [100]. Indeed, NK cells are able to recognize their target cells through a complex integration of antagonistic pathways downstream of activating and inhibitory receptors, whose engagement results in the regulation of cell effector functions. This mechanism dictates whether or not NK cells should be activated to kill the target cell and is also responsible for the discrimination between

healthy self-cells and target cells, thus avoiding the destruction of healthy self-components [101, 102]. A very sophisticated “zipper” is generated between NK cells and their partner cells, defining their synaptic contact, and the integration of activating and inhibitory signals governs NK cell function [103].

Since 2009, novel subsets of the innate lymphoid lineage have been identified and characterized and a recent nomenclature has proposed to call the innate lymphocytes, including NK cells, “Innate lymphoid cells” (ILCs) [104]. ILCs were defined by Spits H. in 2012 as cells with “three main features: the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors; a lack of myeloid cell and dendritic cell phenotypical markers; and their lymphoid morphology” [104]. ILCs are divided in two lineages: cytotoxic ILCs that consist of conventional NK cells and 3 subsets of helper-like ILCs (ILC1, ILC2 and ILC3), which mirror the T helper cell profiles [105]. The subset identification was based on genetic reporter systems and it was defined as follows: cNK (IL-7R α ⁻, GATA-3 independent, Eomes⁺); ILC1 (IL-7R α ⁺, GATA-3 dependent, T-bet⁺); ILC2 (IL-7R α ⁺, GATA-3 dependent, GATA-3⁺); ILC3 (IL-7R α ⁺, GATA-3 dependent, ROR- γ t/T-bet⁺). Recent studies have revealed transcriptional and functional plasticity among certain ILC subsets [105, 106]. Smyth’s group has recently shown that tumor infiltrating cNK cells (CD49a⁻CD49b⁺Eomes⁺) can be converted into “intermediate ILC1” (CD49a⁺CD49b⁺Eomes⁺) and ILC1 (CD49a⁺CD49b⁻Eomes⁻) and this mechanism is driven by TGF β signaling [107]. The localization, definition and role of ILC subsets in physiological or pathological conditions need to be further characterized.

2.2.2 NK cell development in mice and humans

Multiparameter immunophenotyping, purification of specific cell subsets, in vitro differentiation systems and gene-modified/-reporter mouse models have played a key role in the characterization of the NK cell development process [108]. Di Santo's and Caligiuri's groups conducted pioneering studies to define the NK cell developmental pathway in mice and humans, respectively (Figure 5) [108, 109]. Both murine and human NK cell differentiation was conventionally divided into stages, although they obviously represent a simplification of a continuous process occurring from hematopoietic stem cells (HSC) to generate mature NK cells. As a result of pluripotency loss in HSC, NK cell committed precursors (NKP) are generated in the bone marrow and fetal thymus and they will give rise to mature NK cells, whereas other lineage options will be no longer available [110, 111].

NK cell commitment

In mice, NK cell committed precursors arise in the bone marrow from lymphoid progenitors, which are able to generate B, T and NK cells in vitro or upon transplant and are defined as early lymphoid progenitors (ELP; Lin⁻cKit⁺Flt3⁺) and common lymphoid progenitors (CLP; Lin⁻cKit^{low}IL-7R α ^{low}Sca-1^{low}Flt3^{low}) [112-114]. It is not clear whether the transit through these intermediate subsets is really necessary for NK cells to develop and mature, since c-Kit-deficient mice, which lack a fundamental molecule for stem cell differentiation, show normal numbers of NKP and mature NK cells [115]. Moreover, it was observed that the thymocyte precursor (CD44⁺CD25⁺) displays NK cell potential under certain conditions and even following Notch signaling, which is essential for T cell development [108, 116].

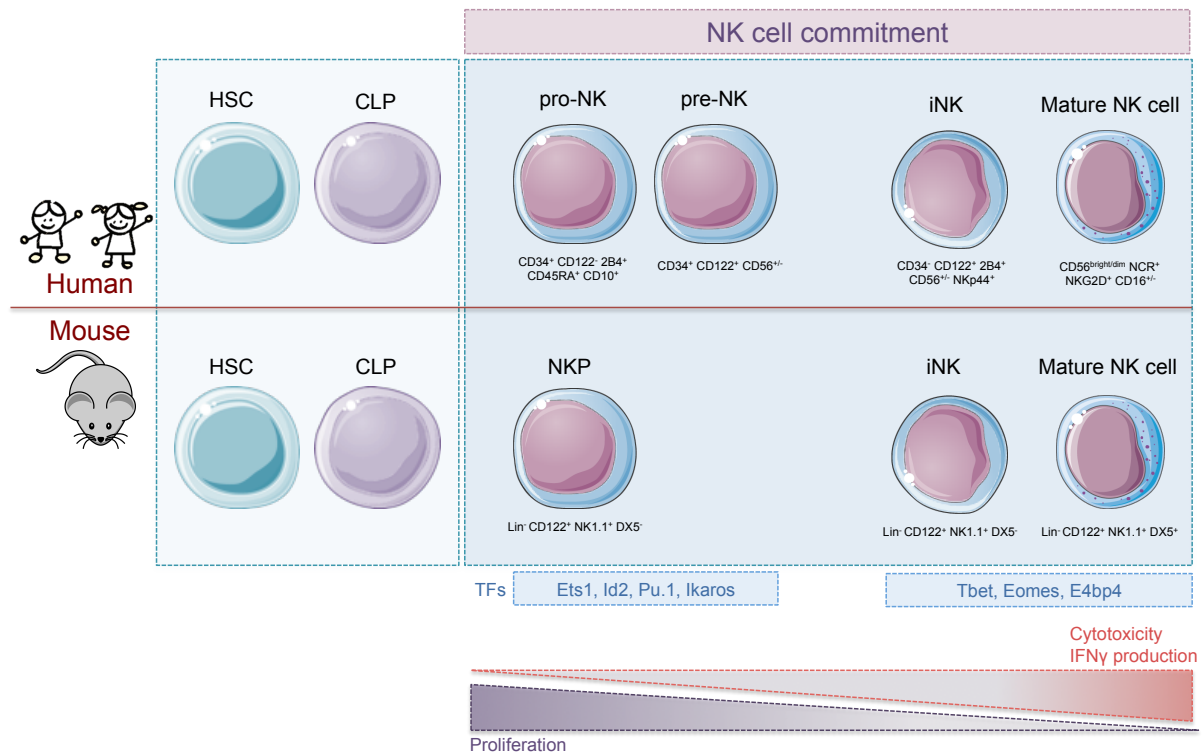


Figure 5. NK cell development.

NK cell precursors and the major markers used to identify them in human and mice are shown (HSC: hematopoietic stem cells; CLP: common lymphoid precursors; pro-NK: NK progenitor; pre-NK or NKP: NK precursor; iNK: immature NK). The main transcription factors involved in early and late NK cell development are: *Ets1*, *Id2*, *Pu.1*, *Ikaros*, *Tbet*, *Eomes* and *E4bp4*. During the differentiation process NK cell lose the proliferative capacity and acquire a cytotoxic and cytokine producing potential.

NK cell commitment appears to be dependent on CD122 (IL-2R β) expression, allowing IL-15 responsiveness and indeed, NKP are defined as Lin⁻CD122⁺NK1.1⁺DX5⁻ [117]. Interestingly, the differentiation of NKP from HSC is not dependent on IL-15, but it relies on other molecular signals that regulate CD122 expression, which are not fully characterized (they could include IL-7, Flt3L, c-KitL, IL-21) [118-120]. NKP were shown to have the potential to generate mature NK cells with a frequency of one in eight, in vitro [117].

In humans, NK cell differentiation studies were mainly performed as ex vivo analyses of CD34⁺ populations and collectively led to the proposal of a sequential model of early NK cell development, based on the surface expression of CD34, c-Kit, CD94 and CD16 [101, 109]. In

particular, the IL-15-non-responsive NK cell progenitor (pro-NK; CD34⁺, CD122⁻, CD45RA⁺, CD117⁻ CD10⁺, 2B4⁺) gives rise to the IL-15-responsive NK precursor (pre-NK; CD34⁺, CD122⁺), following stimulation with IL-3, IL-7 and Flt3L, with a frequency of 1 in 3.5 [121, 122]. Pre-NK cells give rise to immature NK (iNK) cells that are committed to the NK cell lineage. Indeed, they lack the expression of antigens associated with T, B and dendritic cells and they express NK-associated markers such as 2B4 and NKp44. However, they do not have the ability to produce IFN- γ and to mediate cytotoxicity [122].

The upstream signals regulating the transition from HSC to NK committed cells are still poorly understood but specific transcription factors are involved and cell-to-cell interaction are needed [108]. Indeed, HSC in liquid culture generate few NK cells, which are enriched in presence of stromal cells. Regarding transcriptional regulation, PU.1 was shown to act at early stages of NK cell development and a sophisticated balance of Id and E-box transcription factors (e.g. Id2 and E2A) determines NK cell fate [123, 124]. Ets-1 and Ikaros were also demonstrated to be critical for NK cell development, but their exact stage-specificity and mechanism of action have not been reported yet [125, 126].

From NKP to mature NK cells

NK committed precursors will then acquire the phenotypical and functional features of differentiated NK cells. In the mouse, Di Santo JP and Vosshenrich CA recently proposed the definition of immature and mature NK cells, derived from NKP, based on the acquisition of CD49b (DX5) [108]. The generation of immature NK cells (iNK; NK1.1⁺, DX5⁻) from NKP in the bone marrow is regulated by the T box transcription factor (Tbx21, encoding T-bet) and possibly the Nuclear factor interleukin-3-regulated protein (Nfil3 or E4bp4) [127, 128]. Mature NK cells (mNK) express DX5 and the transcription factor essential for this terminal step appears to be Eomes [129, 130]. Differentiated NK can be further subdivided in 4-stages

of maturation, based on the expression intensity of CD27, a member of the TNF receptor superfamily, and CD11b: double-negative (DN; CD27⁻CD11b⁻), CD11b^{low} (CD27⁺CD11b⁻), double-positive (DP; CD27⁺CD11b⁺) and CD27^{low} (CD11b⁺CD27⁻). These subsets were shown to be developmentally related and the transit from DN to CD11b^{low} cells is considered the terminal maturation process of NK cells, which involves a decreasing proliferative capacity and increasing cytotoxic activity (Figure 6) [131].

In humans, iNK cells differentiate towards mature NK cells, acquiring CD94-NKG2A, NKG2D and NKp46 expression, and the capacity to produce IFN- γ and kill [122, 132-134]. Mature NK cells are defined by the expression of the lineage marker CD56 and Lanier L. first proposed that CD56^{bright} (CD16^{+/-}CD94⁺) and CD56^{dim} (CD16⁺CD94⁻) NK cells represent two sequential stages of NK cell maturation [135]. In vitro and in vivo evidences largely supported this hypothesis and indeed, CD56^{bright} NK cells express c-Kit and are poorly cytotoxic. Following stem cell in vitro differentiation or in vivo transplantation CD56^{bright} NK cells appear earlier than CD56^{dim} NK cells [136-138]. It was observed that T-bet mRNA expression, which is crucial for murine NK cell development, is induced in human CD56⁺ NK cells (Figure 6) [139].

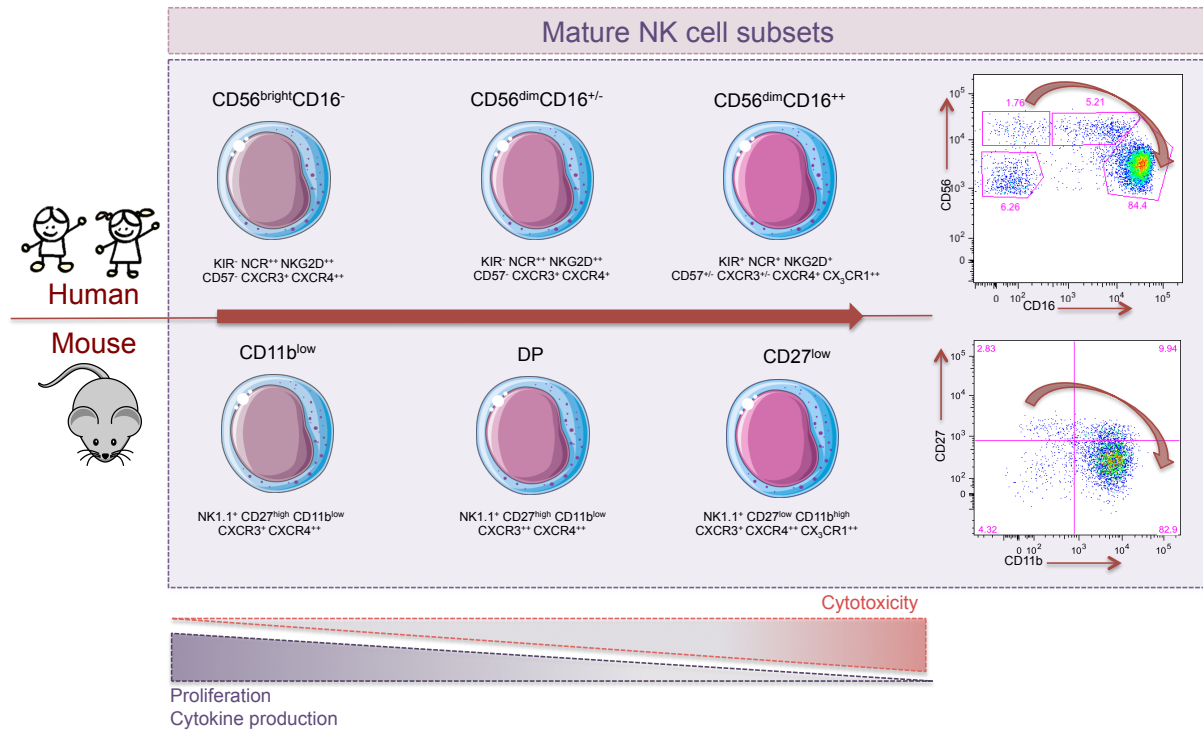


Figure 6. Mature human and murine NK cell subsets.

Mature NK cell subsets and the major markers used to identify them in human and mice are shown. During the late differentiation process NK cells lose the proliferative and cytokine producing capacity and acquire a cytotoxic potential.

2.2.3 NK cell localization and organ-specific features of NK cells

NK cell maturation drives the acquisition of several chemotactic receptors and adhesion molecules, allowing NK cells to migrate from the bone marrow through the blood to spleen, liver, lung, lymph nodes, omentum and uterus during gestation [140, 141]. It is generally accepted that early steps in NK cell development occur within the bone marrow but it is unclear whether final maturation could also be completed in other organs. In this regard, the identification of NK cell precursors in human lymph nodes and immature TRAIL⁺ NK cells in murine liver and spleen could support this possibility [142].

NK cells represent a minor fraction of lymphocytes in most tissues (e.g. in the mouse: 2-3% in spleen, 3-4% in peripheral blood, 0,5-1,5% in bone marrow, 10% in lung and 8-10% in liver; in humans: 2-18% in peripheral blood and 30-50% in liver) [141]. NK cell turnover was shown to be around two weeks in blood, both in mice and humans [143].

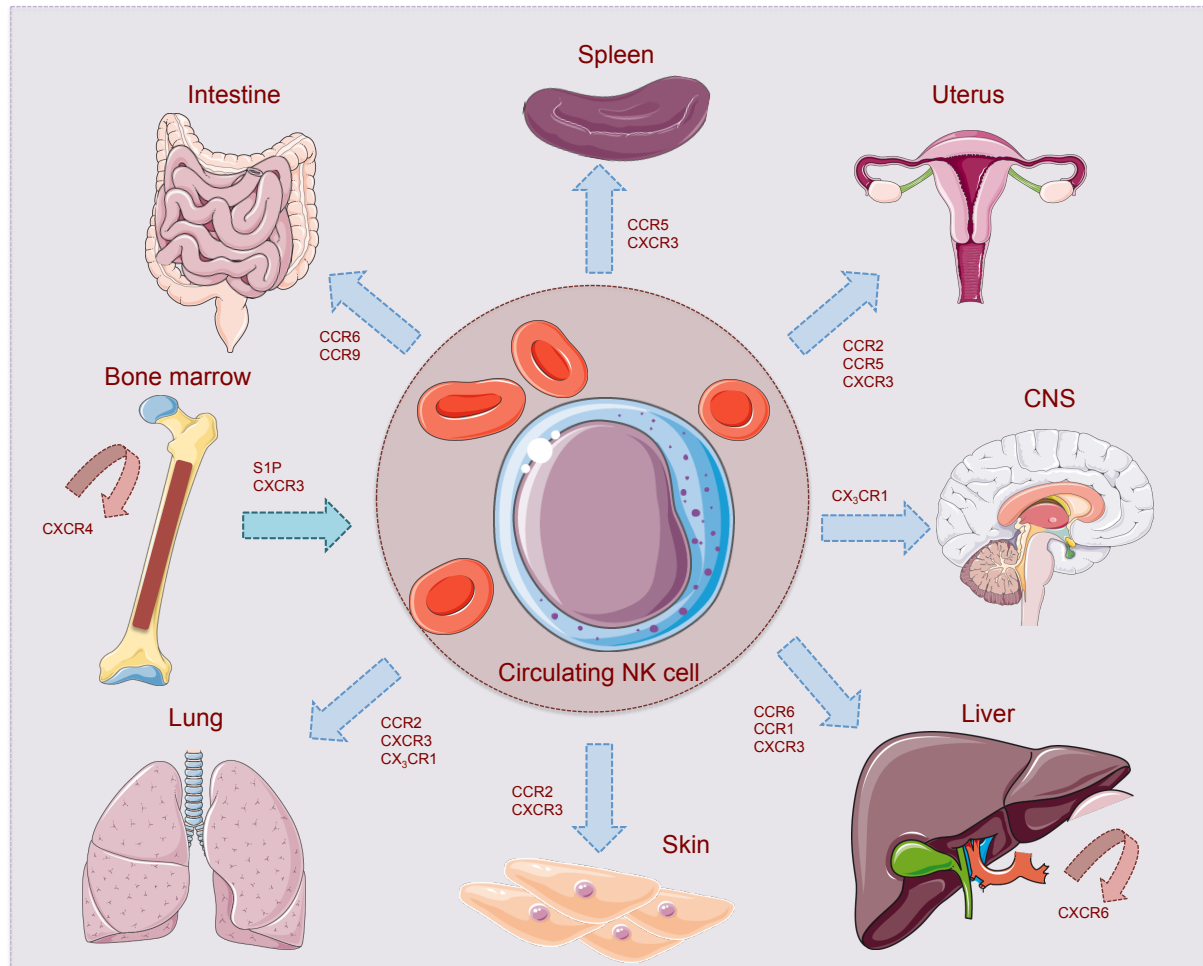


Figure 7. NK cell localization.

BM-derived NK cells exert the bone marrow, circulate through the blood and reach several organs in physiological and pathological conditions. Their migration and localization to different districts is driven by different chemokines-chemokine receptors (indicated in the figure).

NK cells subsets, which are functionally diverse, display distinct homing capacity and localization and therefore organ-specificity. Indeed, in the mouse, immature NK cells ($CD11b^{\text{low}}CD27^{+}$) are mainly found in bone marrow and lymph node, whereas mature NK

cells (CD11b⁺CD27^{low}) are largely present in blood, spleen, liver and lung. The intermediate population (CD11b⁺CD27⁺) is homogenously distributed [144]. Moreover, a subset of B220⁺ CD11c⁺ NK cells was described as enriched in secondary lymphoid structures and it is able to secrete high levels of IFN γ [145]. In humans, the majority of circulating and splenic NK cells displays a mature and potentially active phenotype (CD56^{dim}CD16⁺Perforin⁺). CD56^{bright}CD16⁻ NK cells are considered an earlier stage of NK cell maturation and are predominantly found in tonsils and lymph nodes and produce cytokines (IFN γ , TNF α and GM-CSF) after stimulation with pro-inflammatory factors [146]. Immature NK cells do not express cytoplasmic perforin and they acquire cytotoxic capacity only after sustained and prolonged stimulation. Murine NK cells lack the expression of CD56, making it difficult to compare murine and human NK cells. Nevertheless, there is evidence that immature NK cells in the mouse resemble CD56^{high} human NK cells [103].

Hepatic NK cells

The liver represents a unique immunological landscape, enriched in NK cells, which are preferentially located in the sinusoids and often adhere to endothelial cells [147]. NK cells represent 5-10% and 30-50% of hepatic lymphocytes in mice and humans, respectively and certain pathological conditions, such as viral infections, drive a profound accumulation of NK cells in the liver [140, 148]. Hepatic NK cells show distinct phenotypic features compared to mature NK cells and several lines of evidence have suggested that liver NK cells and conventional NK cells arise from different precursors. For instance murine hepatic NK cells express lower levels of maturation markers, such as CD11b, KLRG1 and Ly49 receptors, and higher levels of the apoptosis inducers TNF-related apoptosis-inducing ligand (TRAIL) and FasL. Liver-resident cells produce CCL2, CCL3, CXCL10 and CXCL16 and therefore mediate the recruitment of NK cells expressing CCR1, CCR2, CCR3, CCR5, CXCR3 and

CXCR6 [149, 150]. The specific features of liver-resident NK cells reflect their ability to adapt to specific environments and in support of this, it was shown that adoptively transferred splenic NK cells can migrate to the liver and adopt phenotypic and functional features closely similar to liver resident NK cells [151].

Hepatic NK cells face the task of balancing the immune response against viruses, bacteria, stressed or transformed cells and tolerance to food, self antigens and gut-derived microbiota antigens. At steady-state condition, NK cells promote liver tolerance [147]. Indeed, it was shown that liver NK cells express high levels of the inhibitory receptor NKG2A, compared to systemic NK cells. LPS and (lipoteichoic acid) LTA that arrive in the liver activate Kupffer cells, triggering the release of IL-10 and the induction of NKG2A in NK cells, which may contribute to NK cell-mediated liver tolerance. NKG2A engagement in hepatic NK cells is indeed responsible for the promotion of a tolerogenic phenotype of dendritic cells [140, 152]. In the inflamed liver, IFN α , IFN γ , IL-2, IL-6, IL-12, IL-15 and IL-18, produced by Kupffer cells, NKT cells and other liver resident cells, contribute to the activated phenotype of NK cells [152]. Hepatic NK cells are crucial mediators of the innate resistance to several hepatic viruses, such as hepatitis B and C virus (HBV and HCV), which is favored by liver-resident specific features of NK cells. Viral PAMPs activate Kupffer cells, which produce IL-6 and IL-18 and in turn trigger the activation of NK cells, boosting their killing capacity. In hepatic NK cells, increased expression of NKp30 was shown to promote the control of HBV and IFN α -induced expression of TRAIL might be associated with HCV clearance. In this regard, liver NK cells play a key role in the therapeutic activity of IFN α treatment in HBV and HCV patients [153-155]. Following viral infection, hepatic NK cells can acquire a memory phenotype, retaining antigen-specific memory in the absence of viral persistence, which depends on CXCR6 expression [154].

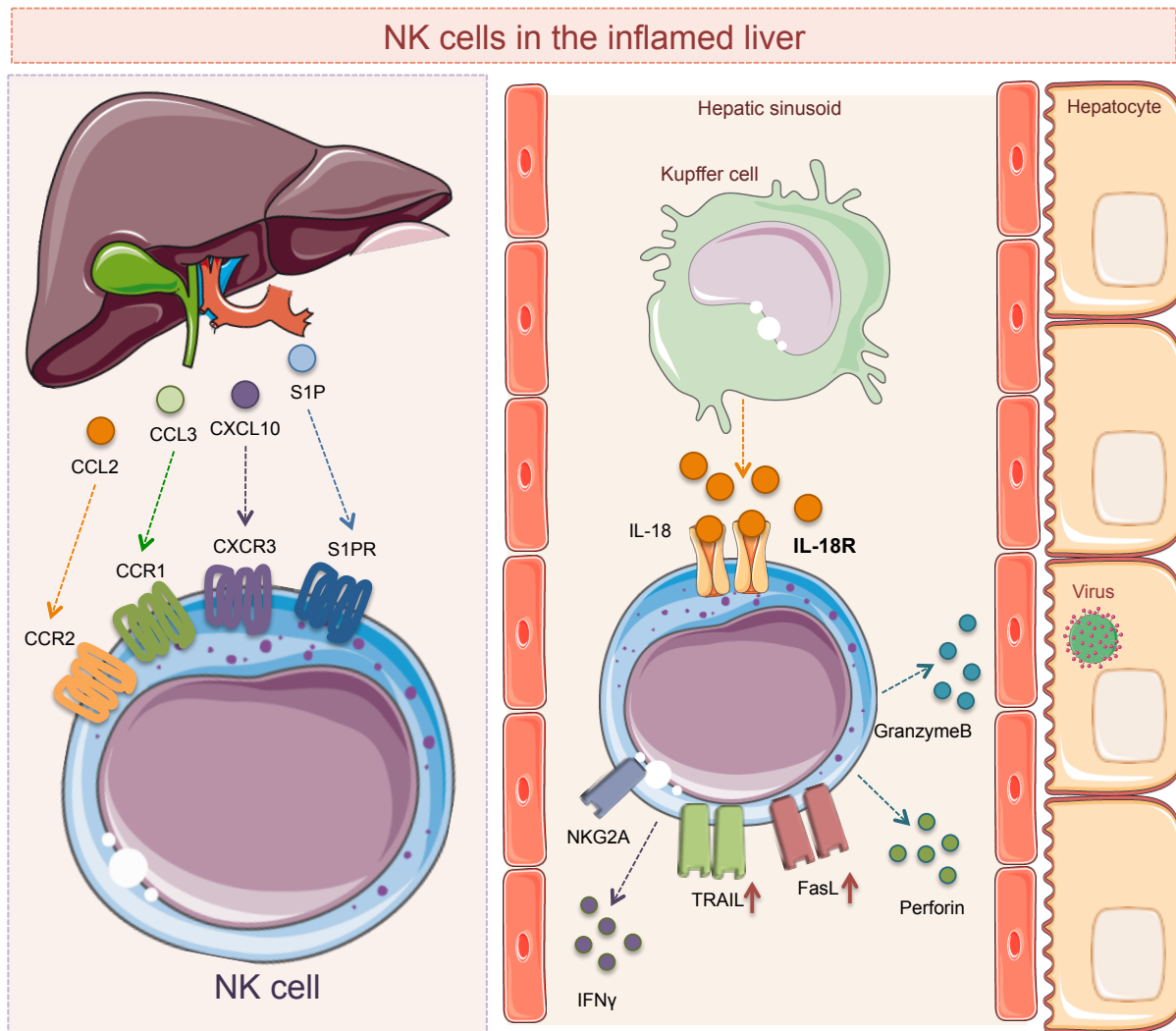


Figure 8. Hepatic NK cells.

NK cells migrate and then reside in the liver through different chemokine receptors (CCR2, CCR1, CXCR3) and S1PR. The liver is particularly enriched with NK cells, which display a peculiar liver-specific phenotype: they express high levels of NKG2A, TRAIL and FasL. Kupffer cells produce high levels of IL-18, which induces NK cell activation.

In chronic liver disease, NK cells have been suggested to favor tissue damage and viral persistence and inhibit liver fibrosis and tissue regeneration mediated by pericytes (hepatic stellate cells; HSC). Indeed, activated NK cells can kill hepatocytes and hepatic stellate cells, resulting in liver injury and wound-healing inhibition [140, 156]. These mechanisms were demonstrated to be dependent on TRAIL- and NKG2D-dependent activation of NK cells. In

chronic HBV- and HCV- infected patients, NKG2A^{high} phenotype of NK cells might contribute to facilitate viral persistence [157, 158].

Thus, in physiological conditions, hepatic NK cells are involved in liver tolerance and comprise a memory-like population. In acute viral infection, chemokines produced by liver-resident cells drive the further accumulation of NK cells, which contribute to viral control and clearance. Liver NK cell cytotoxicity is strictly regulated to maintain tolerance at steady-state conditions and avoid bystander tissue damage after viral clearance. However, in chronic stages of liver infection, NK cells favor liver injury and pathogen persistence and inhibit liver fibrosis and tissue remodeling.

NK cells in other organs

In mucosal tissues, the difficulty of the discrimination of bona fide conventional NK cells and non NK cell-ILCs has been a major challenge and the role of this cell types in physiological and pathological conditions need to be further elucidated. Nevertheless, NK cells represent 10% of lymphocytes in the healthy lung and are also present in skin and gut.

Lung NK cell progenitors were shown to differ compared to bone marrow NK cell precursors and mature lung NK cells express various LY49 receptors and produce higher amounts of TNF, in case of *S.aureus* infection. During acute lung infections, NK cells are enriched in the broncho-alveolar space and their recruitment is mediated by CCR2, CXCR3 and CX₃CR1 [159].

In skin lesions of psoriatic patients, NK cells are localized in the mid and papillary dermis, account for 5-8% of total infiltrating cells and their homing in the skin is dependent on keratinocyte-derived CCL5 and CXCL10. NK cells in the skin exhibit a CD56^{bright}CD158b⁻ phenotype and secrete high levels of IFN γ [160].

In the intestine, NK cells are located predominantly within the lamina propria and are thought to be involved in the protection against viruses and intracellular pathogens. In humans, a cytotoxic, IFN γ -producing, NKp44⁺CD103⁺CD56⁺CD3⁻ population has been identified in the oral and intestinal mucosa, located within the epithelium [161]. Recently, the characterization of NKp46⁺ROR γ t⁺ ILCs and their plasticity towards NKp46⁺ROR γ t⁻ cells has increased the complexity in the discrimination of intestinal cNK cells and ILCs [162]. Further studies are required to elucidate their phenotypic features and role in gut homeostasis and inflammation [163].

NK cells are present in non-pregnant uterus and abundantly accumulate in the decidua during pregnancy. Uterine NK cells represent 70% of decidual leukocytes in early pregnancy and exhibit a unique phenotype [164, 165]. They are CD56^{bright}CD16⁻, they are poorly cytotoxic but contain cytoplasmic granules and they express higher levels of KIRs and CD94/NKG2 receptors, which recognize HLA-C and HLA-E on trophoblast, respectively [166-168]. During pregnancy, they play a crucial role in arterial remodeling, produce angiogenic factors (e.g. VEGF and PLGF) and induce Treg cells, contributing to the maternal-fetal tolerance [169, 170].

In the central nervous system, there is no evidence on the presence of NK cells in physiological conditions and the integrity of the blood-brain barrier and epithelial blood-cerebrospinal fluid barrier prevents the influx of immune cells [171]. However, NK cells have been detected in the inflamed CNS during infections [172, 173], in glioma-bearing mice, in which NK cells account for the 50% of all CNS leukocytes, and in EAE models [174, 175]. NK cell recruitment in the CNS depends on CX₃CL1 secreted by neurons and CCL2 and CXCL10 produced by microglia, astrocytes and other inflammatory cells [176, 177]. NK cells within the brain play a protective role during viral infections and chronic inflammatory conditions, in which they may have an anti-inflammatory role. In this regard, it was shown

that CNS NK cells express high levels of the inhibitory receptor NKG2A and can directly lyse microglia, thus inhibiting the activation of autoimmune T cells [176, 177]. In the murine EAE model, NK cells negatively regulate CNS inflammation and depletion of NK cells is associated with exacerbated neurological defects [178, 179].

2.2.4 Regulation of NK cell functions

NK cell activation depends on a delicate balance between activating and inhibitory signals and the integration of these pathways prevents NK self-reactivity and governs NK cell activation in presence of cells in “distress” (Table 1, Figure 9) [100, 103]. NK cells, once activated, can be actively cytotoxic through the release of perforin and granzymes and can secrete cytokines, such as IFN γ , thus participating in the shaping of the adaptive immune response [103, 180, 181]. NK cell effector functions also include antibody-dependent cell cytotoxicity (ADCC): NK cells recognize antibody-coated target cells through the Fc γ RIIA (CD16), which is coupled to CD3 ζ and FcR γ polypeptides, bearing the activatory domains ITAM (immunoreceptor tyrosine-based activation motif) [182] [100]. NK cells recognize damaged, stressed, infected or tumor cells, which express ligands interacting with activating NK cell receptors. Stress-induced ligands on host cells, such as human ULBP and MIC or mouse RAE1, H60 and MULT1 molecules can interact with the activating receptor NKG2D on NK cells [183]. Other ligands of activating receptors are viral encoded non self-ligands, which include cytomegalovirus-encoded m157, directly recognized by Ly49H in the mouse, and TLR ligands, even though the direct role of TLRs in NK cells remains an unsettled issue [184-187]. The natural cytotoxicity receptors (NCR), such as NKp46/NCR1, NKp44/NCR2 and NKp30/NCR3, which are linked to ITAM-bearing CD3 ζ , FcR γ or DAP12, are other

potent activating receptors. NKp46 was reported to interact with influenza- and parainfluenza-derived hemagglutinins [188].

NK cell receptors and ligands				
Receptor	Ligands	Expression on other cells	Inhibitory	Activating
KIR (humans)	MHC class I	Effector and/or memory T cells	X	X
Ly49 (mouse)	MHC class I	Effector and/or memory T cells	X	X
NKG2A	HLA-E (humans) and Qa1 (mouse)	Effector and/or memory T cells	X	
NKG2C	HLA-E (humans) and Qa1 (mouse)	Effector and/or memory T cells		X
NKG2D	MICA, MICB, ULBP1-6 (human) and RAE1a-e, H60a-c, MULT-1 (mouse)	T cells, activated macrophages		X
DNAM1	PVRL2, PVR	T cells and monocytrs		X
TIGIT	PVRL2, PVR	T cells	X	
Tactile	PVR	T cells	X	
FcγRIIIA	Fc domain of IgG	Activated monocytes and macrophages		X
NKp46	HS, HN, HA, vimentin, PfEMP1, bacterial components	T cell subsets and ILC3		X
NKp44	HS, PCNA, KNp44L, HN, HA, viral glycoproteins, bacterial components	T cell subsets, ILC3, pDCs		X
NKp30	B7-H6, HS, BAT3, HA, HCMV pp65, PfEMP1	T cell subsets and epithelial cells		X
PD1	PDL1, PDL2	Activated T and B cells and myeloid cells	X	
4-1BB	4-1BBL	T cells, DCs and endothelial cells		X

Table 1. NK cell receptors and ligands.

NK cells receptors and their ligands expressed by other cell types are listed.

NK cell response also depends on the cytokine microenvironment and the interaction with other immune cells, such as dendritic cells, macrophages and T cells. IL-12, IL-18, IL-15 and type I IFN are strong activators of NK cell effector functions and IL-2 favors NK cell proliferation and activation. CD4 T cell mediated-production of IL-2 in lymph nodes, DC and macrophage-derived IL-18 and IL-15 activate NK cells, whereas T regulatory cell-derived TGF β negatively regulates NK cell function. It has been appreciated that, despite their original definition of natural killers, NK cells do require “priming” to gain a full activation state. IL-15 and IL-18 are well-described mediators of NK cell priming in both steady state and inflammatory conditions [189-195].

NK cell inhibitory receptors prevent NK cell autoreactivity while allowing recognition and killing of stressed target cells. NK cells express several MHC class I-specific inhibitory receptors that include the lectin-like Ly49 dimers in the mouse, the killer cell immunoglobulin-like receptors (KIRs) in humans and the CD94-NKG2A heterodimers in both species, all sharing the intra-cytoplasmic inhibitory domains ITIMs (immunoreceptor tyrosine-based inhibition motifs) [196, 197]. Other NK cell inhibitory receptors that do not recognize MHC class I molecules are 2B4 and NKR-P1A in the mouse and NKR-P1B in humans [102, 198]. NK cells can detect the lack of MHC class I occurring in viral infected or tumor cells and this process is called the “missing self” recognition. Thus, healthy cells that express MHC class I molecules and low levels of stress-induced molecules are protected from NK cell killing, whereas cells “in distress” that up-regulate stress-induced ligands and down-regulate MHC class I molecules are recognized and killed [102, 199, 200].

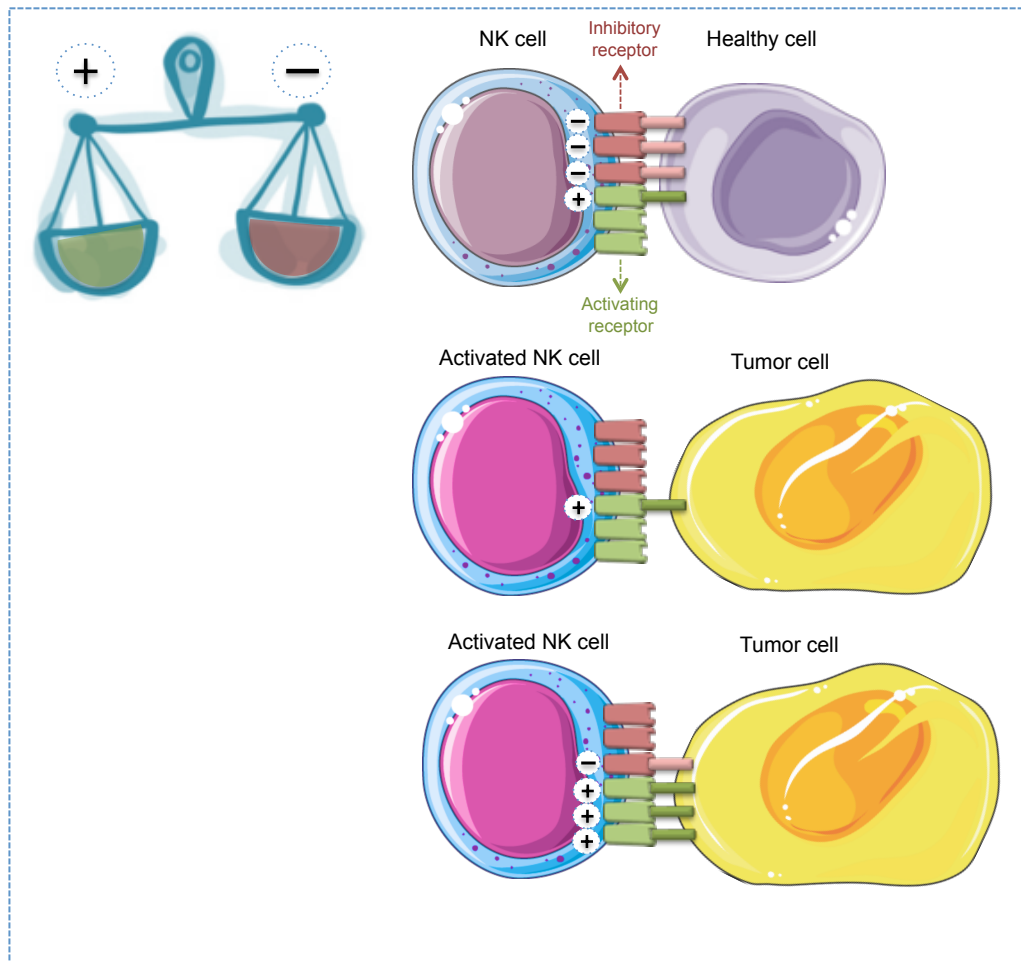


Figure 9. Regulation of NK cell activation.

NK cell activation is dependent on a delicate balance between activating and inhibitory receptors, which engagement drives NK cell inhibition and/or NK cell activation. Healthy cells express inhibitory ligands and do not activate NK cells. Target cells (e.g. tumor cells) can down-modulate inhibitory ligands and upregulate or de novo express activating ligands, therefore activating NK cell response.

NK cell education

The acquisition of NK cell tolerance to self depends on the expression of MHC class I specific-inhibitory receptors and on the “education” or “licensing” system. NK cell education occurs during NK cell development and is based on the need to avoid auto-reactivity, ensuring the generation of self-tolerant killer cells [201-203]. Since NK cell receptors do not undergo somatic recombination, their potential for autoreactivity is due to the fact that the expression pattern of MHC class I receptors is largely random. Some NK cells lack inhibitory receptors that recognize MHC class I and/or express activating receptors that recognize self ligands, including MHC molecules [196]. During the education process, NK cells that lack self MHC-specific inhibitory receptors become hyporesponsive, whereas NK cells that express MHC receptors are responsive to stimulation but tolerant to self cells, which indeed engage the MHC-specific inhibitory receptors. For instance, in mice or humans that lack MHC class I molecules, NK cells fail to kill MHC class-I deficient autologous cells and display reduced responses to other stimulations [204-206]. In humans, NK cells expressing the activating receptor KIR2DS1, which is specific for the HLA-C2, are responsive in HLA-C1/C1 or HLA-C1/C2 donors, but not in HLA-C2 donors [207, 208]. The intensity and quality of NK cell response was shown to be commensurate with the number of different self-MHC inhibitory receptors expressed by the cell. Whether the process of NK cell education depends on the active “arming” (e.g. the induction is mediated by MHC-specific inhibitory receptors) or the active “disarming” (e.g. the induction is mediated by the lack of MHC-specific inhibitory receptors), or both, is still matter of debate [209].

2.2.5 Tumor surveillance mediated by NK cells

NK cells were first reported to be implicated in tumor surveillance around 1980, when defective NK cell function caused by genetic disorders was correlated with a higher incidence of cancer in humans [210]. In agreement, mutant mice with impaired NK cell function or treated with NK cell-depleting antibodies showed increased tumor growth and metastasis [211, 212]. Later, a seminal long-term epidemiological study demonstrated that the susceptibility to various types of cancer increased in patients with impaired NK cell activity [213]. Moreover, NK cell deficiencies in humans correlated with higher rates of malignancy and NK cell function was found to be impaired in cancer patients [214].

However, the reported immunodeficiencies and genetic defects affect other immune cell types in addition to NK cells and the ablation of NK cells in the mouse is not 100% specific. Even though NK cells are able to kill a variety of tumor cell lines in vitro, the real contribution of NK cells in primary solid tumors in physiological settings still remains to be clarified. The importance of NK cell-mediated killing of nascent tumors may be speculated, although it is very difficult to have a formal demonstration of this concept. The current literature suggests that NK cells play a crucial role in the prevention and control of hematological malignancies and metastases, but to what extent they are involved in the control of primary tumor growth is matter of debate, also considering the fact that NK cells represent a minor fraction of the tumor immune infiltrate and have limited prognostic significance compared to other lymphocytes, such as CD8⁺ T cells and T regulatory cells [215, 216].

The interplay between NK cells and tumors

NK cell recognition of transformed cells is based on the altered balance of activating and inhibitory ligands expressed by target cells. NK cell killing activity can be activated by tumor cells that down-regulate MHC class I molecules, possibly because of a selective pressure

mediated by CD8⁺ T cells. Alternatively, NK cells can recognize and eliminate tumors that retain MHC class I expression and express one or several activating ligands, thus overcoming the inhibitory signals [215]. The best characterized ligands expressed on tumor cells able to trigger NK cell activation are the NKG2D and NCR ligands [188, 217]. Indeed, NKG2D-deficient (Klrk1^{-/-}) mice are more susceptible to the development of several types of primary tumors, such as transgene-driven lymphoma and prostate carcinoma and the overexpression of NKG2D ligands is protective in models of transplanted tumors [218-220]. The contribution of NCR signaling in NK cell-mediated control of tumors was assessed by *in vitro* cytotoxicity assays, in which neutralizing monoclonal antibodies against NCRs blocked the killing capacity of NK cells [188]. Moreover, Nkp46-deficient (Ncr1^{-/-}) mice were shown to be more sensitive in transplantable models of lymphoma, Lewis lung carcinoma, B16 melanoma metastasis and glioblastoma [221-223]. Several ligands expressed by tumors have been proposed to engage the NCRs, including heparan sulfates, but the only validated ligand is B7-H6, which binds Nkp30 and is expressed by leukemias, lymphomas, melanomas and carcinomas [224].

In the model of methylcholanthrene (MCA)-induced sarcoma, Klrk1^{-/-} and Ncr1^{-/-} mice do not exhibit any enhanced susceptibility, but NK cell depletion experiments demonstrated a crucial role of NK cells in the tumor control [220, 225]. Indeed, a higher incidence of tumor development was observed in RAG-IL-2 γ c-deficient (Rag2^{-/-}Il2rg^{-/-}) mice, which in addition to B and T lymphocytes lack NK cells, compared to RAG-deficient (Rag2^{-/-}) mice [226]. The NK cell-mediated control of MCA-derived sarcomas was demonstrated to be dependent on IFN γ production, together with the consequent macrophage polarization and activation. Moreover, MCA-induced tumors arising in the absence of NK cells were shown to be more immunogenic when transplanted in wild-type hosts, suggesting an NK-based immunoediting process [226]. In agreement, MCA-driven sarcomas and DMBA-driven papillomas developed

in DNAM1-deficient (Cd226^{-/-}) mice expressed higher levels of the DNAM1 ligand CD155. Evidence for NKG2D-mediated editing was observed in MCA-sarcomas derived from Prf^{-/-} mice and Myc-driven B cell tumors derived from Klrk1^{-/-} mice [142, 220, 227]. Similarly, higher levels of NKp46 ligands, detected with an NKp46-Ig fusion protein, were observed in sarcomas isolated from Ncr1^{-/-} mice [225].

Even though these results suggest a role of NK cells in primary tumor surveillance and tumor immune-editing, which is primarily mediated by adaptive immune cells, the specific contribution of NK cells still needs to be elucidated, since DNAM1, NKG2D and NCRs are also expressed by subsets of NKT, T cells and ILCs. For instance, it was recently shown that NKG2D-deficient mice are more susceptible to the development of diethylnitrosamine (DEN)-induced hepatocellular carcinoma and the major role in tumor control was played by NKG2D⁺ T cells [228].

Tumors have developed strategies to evade the immune response and several mechanisms that enable tumor cells to escape the NK cell-mediated control have been documented. For example, it was shown that tumors can shed the NKG2D ligands to decrease NK cell activation and soluble NKG2DLs were detected in sera of cancer patients, suggesting the potential role of NKG2DLs as diagnostic markers [229-232]. Moreover, soluble factors produced by tumor cells, such as lactate dehydrogenase, induce the expression of NKG2DLs on host myeloid cells [233]. Recently, it was demonstrated that the treatment of tumor-bearing mice with a recombinant high-affinity NKG2DL (MULT-1) could reverse the desensitization of NK cells driven by NKG2DL cleavage and NKG2D engagement by host cells [234]. Other escape mechanisms established by tumors cells are the activation of platelets, which inhibits NK cells, the expression of inhibitory ligands and the production of factors that suppress NK cell maturation and activation, such as TGF β , prostaglandin E₂ (PGE₂), indoleamine 2,3-dioxygenase (IDO), adenosine and IL-10 [235-239].

NK cell subsets in solid tumors and hematological malignancies

Tumorigenesis significantly alters the distribution and functionality of NK cell subsets, which are characterized by extensive heterogeneity and plasticity and may play specific distinct roles during cancer progression [240-242]. Poorly cytotoxic CD56^{high}CD16⁻ NK cells were shown to be the prevalent tumor-infiltrating NK cell subset within different solid tumors, such as breast, melanoma, colon cancer and non-small lung cancer [243, 244]. Unlike peripheral blood CD56^{high}CD16⁻ NK cells, tumor infiltrating NK cells (TINK) express high levels of CD9, CXCR3, have lower cytotoxic capacity and produce VEGF, displaying a tumor-promoting, pro-angiogenic phenotype, similarly to decidual NK cells (dNK) [169, 242]. In breast and lung cancer, the accumulation of poorly cytotoxic CD56^{high}perforin^{low} NK cells was described to be due to a peculiar chemokine milieu within the tumor stromal compartment. Indeed, it was observed an up-regulation of CXCL9 and CXCL10, which recruit CD56^{high} NK cells and a down-regulation of CXCL2, which attracts CD56^{dim} NK cells. In breast cancer patients, a higher frequency of immature and less functional NK cells (CD16⁻CD117^{high}CD27^{high}CD57^{low}) was found in both peripheral blood and advanced mammary tumors. Moreover, NK cell activating receptor expression can be also impaired in cancer patients, favoring the suppression of NK cell activation [245, 246]. For instance, in prostate cancer patients, NK cells exhibited a lower expression of several activating receptors (e.g. NKp46, NKp30, NKG2D, DNAM1 and CD16) and a higher expression of the inhibitory receptor ILT2, with more pronounced alterations in NK cells infiltrating metastases than primary lesions [247].

Impaired NK cell function was also described in acute and chronic leukemia (acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML)), myelodysplastic syndromes (MDS) and multiple myeloma. In AML patients, an enrichment of NCR^{low}CD16⁺KIR⁺ NK cells, which

failed to recognize autologous and allogeneic blasts, was described [248, 249]. In AML and ALL patients, peripheral blood NK cells express lower levels of NKp46 and higher levels of NKG2A and in MDS, AML and MM patients, a lower frequency of NKG2D⁺ and DNAM1⁺ NK cells was observed. [250, 251] Recently, it was described a novel CD56^{low}CD16^{low}NKG2A⁺ NK cell subset in pediatric ALL patients. In healthy donors, this subset was shown to be highly cytotoxic and produce high levels of IFN γ , whereas in leukemic patients it was shown to be functionally impaired [252]. Finally, in the context of CLL and CML, the frequency and the receptor profile of CD56^{low} NK cells were similar to those in healthy donors, but they showed reduced cytotoxic capacity [253].

Collectively, these data demonstrate that several mechanisms, including direct suppression of NK cell activation, modulation of NK cell receptors, NK cell maturation and localization are responsible for NK cell functional impairment in solid tumors and hematological malignancies.

NK cell anti-metastatic activity

Although the role of NK cells in the protection against primary solid tumors is still debated and controversial, NK cells are credited to play a prominent role in the control of the metastatic spread, which is a leading cause of cancer-related death [254]. Indeed, in several types of cancer (e.g. gastrointestinal sarcoma, gastric, colorectal, renal and prostate carcinoma) the frequency of circulating or infiltrating NK cells inversely correlated with the presence of metastases [255]. In agreement, NK cell cytotoxic capacity or NK cell activating receptor expression was associated with good prognosis in multiple cohorts of cancer patients with or at risk of metastases. For example, prostate cancer patients who showed the infiltration of NKG2D^{high}NKp46^{high} NK cells did not develop metastases after surgery, with a follow-up of one year [256]. In several preclinical models, NK cell impairment or depletion

was associated with increased metastatic spread, whereas no difference was observed in primary tumor growth, in mice inoculated with syngeneic cancer cell lines, via the intravenous, intracardiac, subcutaneous, intrasplenic or orthotopic route [218, 257, 258]. Along the same line, treatments that boost NK cell activation were demonstrated to provide protection in murine models of metastases [259-261].

The mechanisms of NK cell elimination of metastases and reasons for the more efficient control of metastases compared to primary tumors are not fully elucidated. It is known that epithelial to mesenchymal transition (EMT) is associated with the upregulation of NKG2D ligands (MICA, MICB and ULBPs) and the loss of inhibitory ligands. For instance, the down-regulation of the KLRG1 ligand E-cadherin, which is a hallmark of EMT, allowing epithelial cells to reduce adhesion and increase motility, renders the tumor cells more susceptible to NK cell killing [262, 263]. Secondly, trafficking and organ-specific enrichment of NK cells is a major determinant in their antimetastatic capacity [254, 264]. At which step or steps of the metastatic cascade NK cells act is still controversial. Indeed, most metastasis models are based on transplanted tumors, which do not recapitulate the early metastatic process and rely on late timepoints, being therefore unable to dissect the contribution of NK cells in the control of each step. In this regard, whether NK cell activity is determinant in the local dissemination, hematogenous spread or tumor growth in the colonized site remains an open issue. On the other hand, the role of NK cells in controlling solid tumors may have been actually underestimated because of the difficulty in setting up models to monitor the early stages of carcinogenesis.

2.2.6 NK cell-based immunotherapy

Targeting NK cells, or the immunosuppressive microenvironment to restore or boost NK cell activity is a promising therapeutic tool and several clinical trials have been recently developed to address this issue. The growing number of studies elucidating the molecular mechanisms of NK cell activation and inhibition, their interplay with tumor cells and their homing within the tumor microenvironment have provided the basis for the establishment of novel encouraging strategies to enhance NK cell antitumor immunity in the clinic (Table 2) [265].

Adoptive NK cell therapy

The first NK cell therapy was reported around 1980 and regarded the infusion of IL-2 activated NK cells, called lymphokine-activated killer (LAK) cells, in cancer patients [210]. This approach was limited by the toxicity of IL-2, partially due to the expansion of Treg cells, which were discovered later. In 2002, Velardi and colleagues observed that AML patients who underwent allogeneic bone marrow transplantation with a KIR/HLA-C mismatch showed relapsed at a lower rate, which was the first clinical evidence of an NK cell-mediated benefit in therapy [266]. In this setting, alloreactive NK cells can efficiently eliminate tumor cells, which lack the proper MHC class I ligands engaged by KIRs, suggesting that NK cells mediate an alloresponse towards AML blasts, without causing graft-versus-host-disease (GVHD) [266].

Therapeutical approaches to target NK cells					
Therapy		Stage	Advantages	Drawbacks	Trial
Adoptive transfer	Autologous NK cells	Clinical trial (or FDA	Safe	Low efficacy	UMIN00000752 7

		approved if part of HSCT)			
	Allogeneic NK cells	Clinical trial (or FDA approved if part of HSCT)	Highly effective in some malignancies in case of KIR-mismatch	Substantial depletion of T cells needed to avoid GVHD	NCT00187096
	NK cell lines	Clinical trials	Easy to expand, safe transfer after irradiation	Low efficacy if not modified	NCT00900809; NCT02465957
	CAR NK cells	Clinical trials	Redirect NK cell activity against a specific tumor antigen	Difficulty to manufacture	NCT0099513; NCT01974479
Cytokines	IL-2	FDA approved	Repeated injections well tolerated at low doses	Toxicity at high doses (capillary leak syndrome, activation of Tregs)	
	IL-15	Clinical trials	Activates both NK cells and CD8 T cells, no activation of Tregs	Potential toxicity (fever, thrombocytopenia and hypotension)	NCT01385423; NCT01875601
	ALT-803 (IL-15SA-IL-15Ra-Su-Fc)	Clinical trial	More potent NK cell activator than IL-15	Potential toxicity	NCT02099539
Anti-cancer agents	IMiDs and imatinib mesylate	FDA approved	Direct anti-tumor effect combined with immunostimulatory effects		
	Bortezomib and genotoxic agents	FDA approved	Direct cytotoxicity toward tumor cells, sensitize tumor cell to NK cell killing	Might have suppressive effects	
	GSK3 inhibitors	Clinical trial	Direct anti-tumor effect, enhances NK cell killing	Might promote Treg cell function	NCT01632306; NCT01287520; NCT01214603
Targeting immune suppression	Depletion of Treg cells	Clinical trial	Improves efficacy of haploidentical NK cell transfer		NCT01106950
	Inhibitors of the TGF- β	Clinical trial and FDA approved	Broad effect on immune responses not restricted to	Effects of TGF- β blockade on tumor	NCT02304419

	pathway	(not for cancer)	NK cells	progression not well defined	
	Inhibitors of the adenosine pathway	Clinical trial	Safe, broad effect on immune responses not restricted to NK cells		NCT02403193
Agonists of NK cell activating receptors	Tumor-targeting mAbs	FDA approved	Redirect NK cell activity against a specific tumor antigen	Possible on-target, off-tumor effect	
	BiKEs and TriKEs	Pre-clinical development	Redirect NK cell activity against a specific tumor antigen	Possible on-target, off-tumor effect	
	mAbs to CD137	Clinical trial	Stimulate NK cell ADCC and T cells	Conflicting reports on the stimulation of human NK cells, severe liver toxicity at high doses	NCT02420938; NCR01775631; NCT01307267; NCT02110082
Checkpoint inhibitors	mAbs to KIRs (IPH2101 and IPH2102)	Clinical trials	Safe, KIR inhibition (can be used in any patient without KIR genotyping)	Low efficacy, needs combination	NCT02399917; NCT01592370; NCT02252263; NCT02599649; NCT02481297; NCT01687387; NCT01714739; NCT01750580
	mAbs to NKG2A (IPH2201)	Clinical trials	NKG2A inhibition (can be used in any patient without KIR genotyping)		NCT02459301; NCT02331875; NCT02557516; NCT02643550; NCT02671435

Table 2. Therapeutic approaches to target NK cells.

The ongoing therapeutic approaches to target NK cells in oncological patients and their advantages and disadvantages are listed.

NK cells for adoptive therapy were later derived from different sources. They can be obtained in either autologous (from the patient) or allogeneic (from a healthy donor) settings [267]. Clinical trials in AML have reported that the treatment with haploidentical NK cells caused complete remissions in patients with poor prognosis and 100% disease-free survival in

a pediatric cohort [266, 268, 269]. Conversely, KIR mismatch treatment is not protective in any other haematological cancer than AML and several studies showed that the transfer of autologous NK cells does not influence the clinical response in patients with renal-cell carcinoma, advanced gastrointestinal cancer and metastatic melanoma [266, 270, 271].

The combination of different cytokines (i.e. IL-2, IL-12, IL-15, IL-18, IL-21) has been tested to activate NK cells before transfer. It was reported that IL-15-stimulated NK cell treatment led to a clinical response in pediatric patients with solid tumors [272, 273]. Ongoing clinical trials have been testing the potential of NK cell transfer in combination with IL-15 in both solid cancer and hematological cancer patients (NCT01385423 and NCT01875601) and anti-CD19 CAR-engineered NK cells in B-ALL patients (NCT00995137 and NCT01974479). In the last few years, the manipulation of NK cells ex-vivo has significantly improved and since the number of circulating NK cells is very low and naïve NK cell cytotoxicity can be enhanced, various protocols have been developed to expand NK cells with a higher antitumor potential. Nevertheless, the promising potential of adoptive transfer therapies with NK cells is still hampered by the high sensitivity to manipulation and cryopreservation and the low transfection efficiency of these cells.

Enhancing NK cell activation and releasing NK cell inhibition

NK cell functionality can be improved by targeting immunosuppressive factors or blocking inhibitory receptors and, on the other hand, by administrating activating ligands or cytokines, or targeting activating receptors. For example, treatment with recombinant IL-15 or IL-15-IL15R α complexes is being tested in metastatic patients in clinical trials (NCT02099539, NCT01385423 and NCT01875601) [274]. Among the inhibitory cytokines, TGF- β is the major suppressor of NK cell functions and a pharmaceutical inhibitor of TGF- β signaling (galunisertib) is being tested in a clinical trial in cancer patients (NCT02304419).

Moreover, NK cells, together with myeloid cells, are involved in ADCC and may contribute to the efficacy of several tumor-targeting monoclonal antibodies (e.g. trastuzumab, cetuximab and rituximab, targeting HER2, EGFR and CD20, respectively) [275, 276]. In this regard, bispecific or trispecific killer-cell engagers (BiKE or TriKE), which bind CD16 and tumor antigens, have been developed to favour the engagement of CD16 and the ADCC response [277, 278]. BiKEs that bind tumor antigens and NKG2D have been also designed and tested in preclinical studies in models of multiple myeloma [279].

A recent “breakthrough” in oncological treatments is the discovery of checkpoint molecules and the development of checkpoint inhibitors [280-288]. In some conditions, activated NK cells can express PD-1 and CTLA4, which are targets of several FDA-approved cancer immunotherapy drugs, originally designed to reverse T cell exhaustion. In multiple myeloma patients, it was observed that NK cells expressed PD-1 and anti-PD-1 treatment reestablished NK cell antitumor activity [289-291]. In addition, Tim-3 expression was demonstrated to correlate with a poor prognosis and Tim-3 blockade restored NK cell activities in melanoma patients [292]. NK cells were also shown to exert ADCC against cancer cells after treatment with an anti-PDL1 monoclonal antibody [293]. The contribution of CTLA4 blockade in boosting NK cell activity is still unclear and might be indirect and dependent on T cell re-activation [294, 295]. Finally, the neutralization of the classical inhibitory receptors of NK cells has been also tested. Indeed, the blocking mAb IPH2101, which recognizes KIR2DL-1, KIR2DL-2 and KIR2DL-3 and therefore blocks KIR-mediated inhibition triggered by HLA-C (both group 1 and 2), had no effect as a single agent in patients with multiple myeloma but it is being currently tested in combination in eight clinical trials in patients with solid and hematological cancer [296-298]. A monoclonal antibody blocking NKG2A (monalizumab) has been recently designed and there are several ongoing clinical trials for the treatment of CLL and various carcinomas [299].

Given the success of checkpoint inhibitors in oncological treatments, the characterization of novel targets or combinations to unleash the antitumor potential of cytotoxic lymphocytes is now crucial.

2.2.7 NK cells and viruses

NK cells play a crucial role in controlling viral infections and indeed, patients with an impaired NK cell number or functionality show recurrent varicella virus, papillomavirus and herpes virus infections [300-305]. The model of murine cytomegalovirus (MCMV) has been widely characterized and used to highlight not only the importance of NK cells in viral clearance, but also to appreciate the molecular mechanisms of NK cell-mediated control of viral spread, NK cell crosstalk with other immune cell types, the viral evasion process and the coevolution of viral species and the immune response. Finally, the MCMV model has highlighted the novel concepts of specificity of NK cells towards certain viral antigens and the consequent expansion of a specific long-lived “memory” NK cell population [300, 306, 307]. The NK cell response to CMV infection, which is a controlled but persistent infection in immunocompetent individuals, occurs early upon infection and drives the expansion, contraction and persistence of a CMV-specific NK cell population, defined as Ly49H⁺ in the mouse and NKG2C⁺ in humans [306, 308-310]. In the murine model, it was clearly demonstrated that NK cell-mediated clearance of viral infected cells is dependent on a specific viral-encoded protein (m157), recognized by the activating receptor Ly49H, which induces IFN γ production and cytotoxicity [300, 311, 312]. Ly49H⁺ cells that persist after the first encounter with the virus are considered “memory” NK cells, which confer specific protection upon re-challenge with CMV, but not with other heterologous infections [309]. In addition, mouse models of herpes simplex virus, simian immunodeficiency virus (SIV) and

influenza infections also unveiled the NK cell-dependent contribution of viral control and the existence of a NK memory population [313-317]. Moreover, the expansion and persistence of CD57⁺NKG2C⁺ “memory” NK cells, which specifically recognize HLA-E on infected cells, was reported in CMV positive individuals [310, 318, 319]. Activating cytokines produced by myeloid cells (i.e. IL-12, IL-18, IL-15) are also crucial to induce NK cell activation. On the other hand, NK cells were shown to favor neutrophil activation via IFN γ and GM-CSF and macrophage, dendritic cell and T cell migration, activation and polarization [320-322]. Finally, NK cells were shown to be able to kill viral-infected immune cells to prevent viral spread. For example, in HIV-1 pregnant patients, decidual NK cells were reported to kill infected macrophages, but the mechanism is not fully elucidated [323]. Further studies are needed to elucidate the interplay of NK cells with other cell types in the delicate balance of viral control and the regulation of immunopathology.

2.2.8 NK cell involvement in autoimmune reactions

Autoimmune diseases exhibit great diversity in clinical symptoms and causative molecular mechanisms but they all share the presence of an autoreactive adaptive response, dependent on B and/or T cell activation against self-antigens [324]. The involvement of NK cells in autoimmune pathologies has been poorly characterized and it is quite controversial. Indeed, mouse models and correlative studies in patients have suggested either a protective or a promoting role of NK cells in autoimmune reactions [324]. In rheumatoid arthritis (RA), synovium-infiltrating NK cells have been detected in patients and they were shown to be mainly CD56^{bright} and express higher levels of IFN γ compared to the circulating counterpart and to favor the activation and differentiation of monocytes, thus promoting the disease progression [325-327]. Moreover, genetic association studies have unveiled correlations of

allelic variations of KIRs, which favor NK cell activation, with the development of systemic lupus erythematosus (SLE), systemic sclerosis, psoriatic arthritis and type 1 diabetes mellitus [328-332]. In addition, an overexpression of NKG2D ligands (MIC molecules) was observed in a model of gluten-sensitive enteropathy and in patients with Crohn's disease and RA, suggesting a possible disruption of NK cell tolerance and an uncontrolled NK cell cytotoxicity [324, 333, 334]. To date, antirheumatic drugs have not specifically targeted NK cells, but they may be involved in the efficacy of therapies with monoclonal antibodies because of the ADCC activity. Indeed, In multiple sclerosis patients, treatment with anti-IL-2R α (daclizumab) was associated with an increased number of circulating CD56^{bright} NK cells, this correlated with positive clinical outcomes and *ex-vivo* cultures showed that NK cells were able to kill autoreactive T cells [335, 336]. Conversely, NK cell deficiency correlated with some clinical manifestations, such as thrombocytopenia and nephritis in SLE patients [325]. NK cells can produce IL-10 in certain conditions and therefore modulate the pathological immune response in rheumatic diseases, even though little is known about IL-10-producing NK cells [337, 338]. Thus, NK cell role in autoimmunity is still controversial and further careful analysis of the role of NK cells in each disease stage, in different tissues and combining genetic and functional studies might help in the development of novel therapeutic strategies.

3. Rational and aim of the work

The general purpose of this investigation was to characterize the role of IL-1R8/TIR8, an IL-1 family receptor, in NK cells. IL-1 family members are key mediators of immunity and inflammation and play a major role in the regulation of innate and adaptive lymphocyte differentiation, polarization and activation. IL-1R8 acts as a negative regulator of ILRs and TLRs and it is therefore a crucial tuner of inflammatory responses. Previous evidences suggested that human NK cells expressed high levels of IL-1R8 and the role of this receptor in NK cells had not been described yet. NK cell activation is tightly regulated by a delicate balance between activating and inhibitory signals, which is crucial to avoid NK cell self-reactivity and NK cell detrimental responses. We hypothesized that IL-1R8 could act as a negative regulator of NK cells and we aimed at characterizing its role in the modulation of NK cell development and effector functions.

Among the inflammatory mediators regulated by IL-1R8, IL-18 was first described as “interferon- γ (IFN- γ)-inducing factor” and it is an essential molecule involved in Th1 and NK cell responses. Moreover, ILR and TLR pathways can be also targeted by IL-1R8 and were shown to contribute to NK cell activation, even though their direct functionality in NK cells is still matter of debate. For instance, IL-1 β was shown to be involved in human NK cell development and activation and microbiota-derived TLR agonists were shown to contribute to NK cell differentiation. Thus, we first dissected the role of IL-1R8 in NK cell regulation and the mechanism responsible for this regulation. Since NK cells are fundamental players in tumor surveillance and viral control, we then assessed the relevance of IL-1R8-mediated regulation in pathological contexts. Given the promising strategies in oncological treatments acting on inhibitory pathways of the immune system, the characterization of novel inhibitory

pathways in cytotoxic lymphocytes such as NK cells, may have important clinical applications.

4. Methods

4.1 Animals

All female and male mice used were on a C57BL/6J genetic background and 8-12 weeks-old, unless specified. Wild-type mice were obtained from Charles River Laboratories, Calco, Italy or were littermates of *Il1r8*^{-/-} mice. IL-1R8-deficient mice were generated as described [30]. *Il1r1*^{-/-} mice were purchased from The Jackson Labs, Bar Harbor ME, USA. All colonies were housed and bred in the SPF animal facility of Humanitas Clinical and Research Center in individually ventilated cages. *Il1r1*^{-/-}/*Il1r8*^{-/-} mice were generated by crossing *Il1r1*^{-/-} and *Il1r8*^{-/-} mice. *Il18*^{-/-}/*Il1r8*^{-/-} were generated by crossing *Il18*^{-/-} and *Il1r8*^{-/-} mice. Mice were randomized based on sex, age and weight.

Procedures involving animals handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (D.L. N.116, G.U., suppl. 40, 18-2-1992 and N. 26, G.U. March 4, 2014) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). The study was approved by the Italian Ministry of Health (approvals n. 43/2012-B, issued on the 08/02/2012 and n. 828/2015-PR, issued on the 07/08/2015). All efforts were made to minimize the number of animals used and their suffering. In most *in vivo* experiments, the investigators were unaware of the genotype of the experimental groups. Sample size was defined in order to detect differences of 20% or greater between the groups (10% significance level and 80% power).

4.2 Human primary cells

Human peripheral mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors, upon approval by Humanitas Research Hospital Ethical Committee. PBMCs were obtained through a Ficoll density gradient centrifugation (GE Healthcare Biosciences). NK cells were then purified by a negative selection, using a magnetic cell-sorting technique according to the protocols given by the manufacturer (EasySep™ Human NK Cell Enrichment Kit, Stem Cell Technology). Human monocytes were obtained from peripheral blood of healthy donors by two-step gradient centrifugation, first by Ficoll and then by Percoll (65% iso-osmotic; Pharmacia, Uppsala, Sweden). Residual T and B cells were removed from monocyte fraction by plastic adherence. Monocytes were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Pen/Strept and 100 ng/ml M-CSF (PeproTech) for 7 days in order to generate resting macrophages. T and B cells were obtained from peripheral blood of healthy donors using RosetteSep™ Human T Cell Enrichment Cocktail and RosetteSep™ Human B Cell Enrichment Cocktail (Stem Cell Technology), following the manufacturer's instructions. Neutrophils were enriched from Ficoll-isolated granulocytes, using EasySep™ Human Neutrophil Enrichment Kit (StemCell Technologies), according to the manufacturer's instructions.

To analyse pluripotent haematopoietic stem cells (HSC) and NK cell precursors (NKP), human Bone Marrow mononuclear cells were collected from Humanitas Biobank, upon approval by Humanitas Research Hospital Ethical Committee (Authorization 1516, issued on February 26, 2016). Frozen samples were thawed and vitality was assessed by trypan blue and Aqua LIVE/Dead-405 nm staining (Invitrogen), before flow cytometry analysis.

Informed consent was obtained from all subjects.

4.3 FACS analysis

Single-cell suspensions of BM, blood, spleen, lung and liver were obtained and stained. Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used for intracellular staining of Granzyme B and Perforin. Cytofix/Cytoperm (BD Biosciences) was used for intracellular staining of IFN γ . Liver ILC1 were identified as NK1.1⁺CD3⁻CD49a⁺CD49b⁻ cells. Formalin 4% and Methanol 100% were used for intracellular staining of IRAK4, pIRAK4, pS6 and JNK. The following murine antibodies were used: CD45-BV605, -BV650 or -PerCp-Cy5.5 (Clone 30-F11); CD45.1-BV650 (Clone A20); CD45.2-APC, -BV421 (Clone 104); CD3e-PerCP-Cy5.5 or -APC (Clone 145-2C11); CD19-PerCP-Cy5.5, -eFluor450 (Clone 1D3); NK1.1-PE, -APC, -eFluor450 or -Biotin (Clone PK136); CD11b-BV421, -BV450, -BV785 (Clone M1/70); CD27-FITC or -APC-eFluor780 (Clone LG.7F9); CD4-FITC (Clone RM 4-5); CD8-PE (Clone 53-6.7); KLRG-1-BV421 (Clone 2F1); NKG2D-APC (Clone CX5); DNAM-1-APC (Clone 10E5); Ly49H-PECF594 (Clone 3D10); Granzyme B-PE (Clone NGZB); Perforin-PE (Clone eBioOMAK-D); IFN γ -Alexa700 or -APC (Clone XMG1.2); CD107a-Alexa647 (Clone 1D4B); FasL-APC (Clone MFL3); Lineage Cell Detection Cocktail-Biotin; Sca-1-FITC (Clone D7); CD117-PE or -Biotin (Clone 3C11); CD127-eFluor450 (Clone A7R34); CD135-APC or -Biotin (Clone A2F10.1); CD244-PE (Clone 2B4); CD122-PE-CF594 (Clone TM-Beta1); CD49b-PE-Cy7 or Biotin (Clone DX5), CD49a-APC (Clone Ha31/8), from BD Bioscience, eBioscience, BioLegend or Miltenyi Biotec. The following human antibodies were used: CD56-PE (Clone CMSSB); CD3-FITC (Clone UCHT1); CD16-Pacific Blue (Clone 3G8); CD34-PE-Vio770 (Clone AC136); CD117-BV605 (Clone 104D2); NKp46-BV786 (Clone 9E2/NKp46); CD45-PerCP (Clone 2D1); CD19-APC-H7 (Clone SJ25C1); CD14-APC-H7 (Clone M5E2); CD66b-APC-

Vio770 (Clone REA306), from BD Bioscience, eBioscience or Miltenyi Biotec. Biotinylated anti-hSIGIRR (R&D Systems) and Streptavidin-Alexa647 (Invitrogen™) were used to stain IL-1R8 in human cells. Human NKT cells were detected using PE-CD1d tetramers loaded with α GalCer (ProImmune, Oxford, UK). Antibodies to detect protein phosphorylation were as follows: p-IRAK4 Thr345/Ser346 (Clone D6D7), IRAK4, p-S6-Alexa647 Ser235/236 (Clone D57.2.2E); p-SAPK/JNK Thr183/Tyr185 (Clone 81E11), from Cell Signaling Technology. A Goat anti-Rabbit-Alexa647 secondary antibody (Invitrogen™) was used to stain p-IRAK4, IRAK4 and p-SAPK/JNK. Results are reported as mean fluorescence intensity (MFI) normalized on isotype control or fluorescence minus one (FMO). Cell viability was determined by Aqua LIVE/Dead-405 nm staining (Invitrogen) or Fixable Viability Dye (FVD) eFluor® 780 (eBioscience), negative cells were considered viable. Cells were analyzed on LSR Fortessa or FACSVerse (BD Bioscience). Data were analyzed with FlowJo software (Treestar).

4.4 Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's recommendations. RNA was further purified using miRNeasy RNA isolation kit (QIAGEN) or Direct-zol™ RNA MiniPrep Plus (Zymo Research). cDNA was synthesized by reverse transcription using High Capacity cDNA archive kit (Applied Biosystems) and quantitative real-time PCR was performed using the SybrGreen PCR Master Mix (Applied Biosystems) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). PCR reactions were carried out with 10 ng of DNA. Data were analyzed with the Δ^2CT method. Data were normalized based on GAPDH, β actin or 18S expression, as indicated, determined in the same sample. Analysis of all samples was performed in duplicate. Primers were designed according to the

published sequences and listed as follows: *s18/S18*: forward 5'-ACT TTC GAT GGT AGT CGC CGT-3', reverse 5'- CCT TGG ATG TGG TAG CCG TTT-3'; *Gapdh/GAPDH*: forward 5'-GCA AAG TGG AGA TTG TTG CCA T-3', reverse 5'-CCT TGA CTG TGC CGT TGA ATT T-3'; *β actin/ β ACTIN*: forward 5'- CCC AAG GCC AAC CGC GAG AAG AT-3', reverse 5'- GTC CCG GCC AGC CAG GTC CAG -3'; *illr8*: forward 5'- AGA GGT CCC AGA AGA GCC AT-3', reverse 5'- AAG CAA CTT CTC TGC CAA GG-3'; *IL1R8*: forward 5'- ATG TCA AGT GCC GTC TCA ACG -3', reverse 5'- GCT GCG GCT TTA GGA TGA AGT-3'; *illr1*: forward 5'- TGC TGT CGC TGG AGA TTG AC -3', reverse 5'- TGG AGT AAG AGG ACA CTT GCG AA -3'; *illr2*: forward 5'- AGT GTG CCC TGA CCT GAA AGA -3', reverse 5'- TCC AAG AGT ATG GCG CCC T -3'; *illr3*: forward 5'- GGC TGG CCC GAT AAG GAT -3', reverse 5'- GTC CCC AGT CAT CAC AGC G -3'; *illr4*: forward 5'- GAA TGG GAC TTT GGG CTT TG -3', reverse 5'- GAC CCC AGG ACG ATT TAC TGC -3'; *illr5*: forward 5'- GCT CGC CCA GAG TCA CTT TT -3', reverse 5'- GCG ACG ATC ATT TCC GAC TT -3'; *illr6*: forward 5'- GCT TTT CGT GGC AGC AGA TAC -3', reverse 5'- CAG ATT TAC TGC CCC GTT TGT T -3'; *16S*: forward 5'- AGA GTT TGA TCC TGG CTC AG -3', reverse 5'- GGC TGC TGG CAC GTA GTT AG -3'.

4.5 Purification of murine leukocytes

Splenic NK cells and bone marrow neutrophils were MACS enriched according to manufacturer's instructions (Miltenyi Biotec). Purity of NK cells was about 90% as determined by FACS. Purity of neutrophils was $\geq 97.5\%$. NK cells were stained (CD45-BV650, NK1.1-PE, CD3e-APC, CD11b-BV421, CD27-FITC) and sorted on a FACS Aria cell sorter (BD Bioscience) to obtain high purity NK cells and NK cell populations (CD11b^{low}CD27^{low}, CD11b^{low}CD27^{high}, CD11b^{high}CD27^{high} and CD11b^{high}CD27^{low}). Splenic

B and T lymphocytes were stained (CD45-PerCP, CD3e-APC, CD4-FITC, CD8-PE, CD19-eFluor450) and sorted. Purity of each population was $\geq 98\%$. Resulting cells were processed for mRNA extraction or used for adoptive transfer or co-culture experiments. *In vitro*-derived macrophages were obtained from bone marrow total cells. Bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Pen/Strept and 100 ng/ml M-CSF (Peprotech) for 7 days in order to generate resting macrophages. Bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Pen/Strept and 20 ng/ml GM-CSF (Peprotech) for 7 days in order to generate DCs.

4.6 Confocal microscopy

Murine splenic NK cells were MACS enriched, let adhere on poly-D-Lysine (Sigma-Aldrich) coated coverslips, fixed with 4% PFA, permeabilized with 0.1% Triton X-100, incubated with blocking buffer (5% normal donkey serum (Sigma-Aldrich), 2% BSA, 0.05% Tween). Cells were then stained with biotin-conjugated goat polyclonal anti-SIGIRR antibody or biotin-conjugated normal goat IgG as control (both R&D Systems) (10 μ g/ml) followed by Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (Molecular Probes) and DAPI (Invitrogen). Coverslips were mounted with the antifade medium FluorPreserve Reagent (EMD Millipore) and analyzed with an Olympus Fluoview FV1000 laser scanning confocal microscope with oil immersion lens 40 \times (N.A.1.3).

4.7 Stimulated emission depletion (STED) microscopy

Human NK cells were enriched and let adhere on poly-D-Lysine (Sigma-Aldrich) coated coverslips, stimulated with IL-18 (50 ng/ml; 1 min, 5 min, 10 min), fixed with 4% PFA, incubated with 5% normal donkey serum (Sigma-Aldrich), 2% BSA, 0.05% Tween in PBS2+ (pH 7.4) (blocking buffer), and then with biotin-conjugated goat polyclonal anti-human IL-1R8 antibody or biotin-conjugated normal goat IgG (all from R&D Systems) and mouse monoclonal anti-IL-18R α (Clone 70625; R&D System) or mouse IgG1 (Invitrogen), all diluted at 5 μ g/ml in blocking buffer, followed by Alexa Fluor 488– conjugated donkey anti-goat IgG antibody and Alexa Fluor 555 donkey anti-mouse IgG antibody (both from Molecular Probes). Mowiol was used as mounting medium. STED xyz images were acquired in a unidirectional mode with a Leica SP8 STED3X confocal microscope system. Alexa Fluor 488 was excited with a 488nm Argon Laser and emission collected from 505 to 550 nm applying a gating between 0.4 to 7ns to avoid collection of reflection and autofluorescence. Alexa Fluor 555 was excited with a 555/547nm-tuned white light laser (WLL) and emission collected from 580 to 620 nm. Line sequential acquisition was applied to avoid fluorescence overlap. The 660nm CW-depletion laser (80% of power) was used for both excitations. Images were acquired with Leica HC PL APO 100x/1.40 oil STED White objective at 572.3mAU. CW-STED and gated CW-STED were applied to Alexa-488nm and Alexa Fluor 555, respectively. Collected images were de-convolved with Huygens Professional software.

4.8 3'-mRNA Sequencing and Analysis

Splenic NK cells (from 6 mice per genotype and pooled in pairs) were purified as described above and stimulated with IL-18 (MBL) (20 ng/ml for 4 h). RNA was prepared as described

above. The QuantSeq 3'mRNA-seq Library Prep Kit for Illumina (Lexogen) was used to generate libraries, which were sequenced on the NextSeq (Illumina; 75 bp PE). The fastq sequence files were assessed using the fastqc program. The reads were first trimmed using bbdduk in the bbmap suite of software to remove the first 12 bases and a contaminant kmer discovery length of 13 was used for contaminant removal. Regions of length 20 or above with average quality of less than 10 were trimmed from the end of the read. The reads were then trimmed to remove trailing polyG and polyA runs using cutadapt and the quality of the remaining reads reassessed with fastqc. The trimmed reads were aligned to the mm10 genomic reference and reads assigned to features in the mm10 annotation using the STAR program [339]. Differential expression analysis was performed using the generalized linear model (GLM) functions in the R/bioconductor [340] edgeR package [341] with TMM normalization. Gene set analysis was performed using the romer function in the R/bioconductor package limma [342, 343]. Metascape (<http://metascape.org>) was used to enrich genes for GO biological processes, KEGG Pathway and Reactome Gene Sets.

4.9 Measurement of cytokines

BD Cytometric Bead Array (CBA) mouse inflammation kit (BD) or DuoSet ELISA kits (R&D System) were used to measure cytokines.

4.10 *In vitro* functional assays

Total murine splenocytes or enriched murine or human NK cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) 1% L-Glutamine, 1% Pen/Strept and treated with IL-2, IL-12, IL-15 (Peprotech), IL-18 (MBL), IL-1 β (Peprotech) and PMA-

Ionomycin (Sigma-Aldrich), as specified. FasL expression was evaluated upon treatment for 45 minutes with IL-18 (50 ng/ml), IL-15 (50 ng/ml), IL-2 (20 ng/ml) and IL-12 (10 ng/ml). IFN γ production was analysed upon 16 hours of treatment with IL-12 (20 ng/ml) and IL-18 (20 ng/ml) or IL-1 β (20 ng/ml), by intracellular staining using BD Cytfix/CytopermTM Fixation/Permeabilization Kit, following the manufacturer's instructions, or by ELISA. Granzyme B and Perforin intracellular staining was performed upon 18 hours of stimulation with IL-12 (10 ng/ml), IL-15 (10 ng/ml) and IL-18 (50 ng/ml), using Foxp3/Transcription Factor Staining Buffer Set (eBioscience). CD107a-Alexa647 antibody was added during the 4-hour culture and analysed by flow cytometry. BD GolgiPlugTM (containing Brefeldin) and BD GolgiStopTM (containing Monensin) were added 4 hours prior to intracellular staining. PMA (50 ng/ml)- Ionomycin (1 μ g/ml) were added 4 hours prior to intracellular staining, when specified.

NK-DC co-culture experiments were performed as previously described [193]. DCs were treated with LPS from Escherichia coli O55:B5 (Sigma-Aldrich; 1 μ g/ml) or CpG ODN 1826 (Invivogen; 3 μ g/ml) and with anti-mIL-18 neutralizing antibody (BioXCell, Clone YIGIF74-1G7; 5 μ g/ml) or Rat Isotype Control (BioXCell, Clone 2A3).

IFN γ and CD107a expression upon viral infection was analyzed by flow cytometry upon 4-hour treatment with BD GolgiPlugTM, BD GolgiStopTM and IL-2 (500U/ml).

Phosphorylation of IRAK4, S6 and JNK was analyzed upon 15-30 minutes of stimulation with IL-18 (10 ng/ml).

4.11 Human primary NK cell transfection

Human NK cells were enriched from peripheral blood of healthy donors and transfected with DharmaconTM AcellTM siRNA (GE Healthcare) using AccellTM delivery medium (GE

Healthcare), following the manufacturer's instructions. 1 μ M SIGIRR-specific siRNA (On-Target Plus; Dharmacon, GE Healthcare) comprised 250 nM of the four following antisense sequences: I, AGU UUC GCG AGC CGA GAU CUU; II, UAC CAG AGC AGC ACG UUG AUU; III, UGA CCC AGG AGU ACU CGU GUU; IV, CUU CCC GUC GUU UAU CUC CUU (all 5' to 3').

4.12 Generation of bone marrow chimeras

Il1r8^{-/-} and *Il1r8*^{+/+} mice were lethally irradiated with a total dose of 900 cGy. 2 h later, mice were injected in the retro-orbital plexus with 4×10^6 nucleated bone marrow cells obtained by flushing of the cavity of freshly dissected femurs from wild type or *Il1r8*^{-/-} donors. Competitive bone marrow chimeric mice were generated by reconstituting recipient mice with 50% CD45.1 *Il1r8*^{+/+} and 50% CD45.2 *Il1r8*^{-/-} bone marrow cells. Recipient mice received gentamycin (0.8 mg/ml in drinking water) starting 10 days before irradiation and for 2 weeks after irradiation. NK cells of chimeric mice were analyzed 8 weeks after bone marrow transplantation.

4.13 Depletion and blocking experiments

Mice were treated intraperitoneally with 200 μ g of specific mAbs (Mouse anti-NK1.1, Clone PK136; Mouse Isotype Control, Clone C1.18.4; Rat anti-mIL-18, Clone YIGIF74-1G7; Rat Isotype Control, Clone 2A3; Rat anti-IFN γ , Clone XMG1.2; Rat IgG1 HRPN; Mouse anti-IL-17A, Clone 17F3; Mouse Isotype Control, Clone MOPC-21; Rat anti-CD4/CD8, Clone GK1.5/YTS; Rat Isotype Control, Clone LTF-2 (all from BioXCell)) and then with 100 μ g

once (anti-NK1.1) or three times (anti-IL-18, anti-IFN γ , anti-IL-17A, anti-CD4/CD8) a week for the entire duration of the experiment.

Anti-NK1.1 treatment was used for the entire duration of the experiment in DEN-induced hepatocellular carcinoma model, which lasts 12-14 months. NK cell depletion was checked in the first months, after 6 months and at sacrifice. NK1.1⁺ cells were depleted, but it should be considered that other NK1.1-expressing cell types (NKT cells and ILC1) are affected by the depletion.

4.14 Microflora depletion

6-week-old mice were treated every day for 5 weeks by oral gavage with a cocktail of antibiotics [ampicillin (Pfizer) 10 mg/ml, vancomycin (PharmaTech Italia) 10 mg/ml, metronidazol (Società Prodotti Antibiotici) 5 mg/ml and neomycin (Sigma-Aldrich) 10 mg/ml]. Control mice were treated with drinking water. A gavage volume of 10 ml/kg body weight was delivered with a stainless steel tube without prior sedation of mice. DNA was isolated from bacterial fecal pellets with PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) and quantified by spectrophotometry at 260 nm. PCR was performed with 10 ng of DNA using the SybrGreen PCR Master Mix (Applied Biosystems) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Data were analyzed with the Δ^2CT method (Applied Biosystems, Real-Time PCR Applications Guide).

4.15 Cancer models

Mice were injected intraperitoneally (i.p.) with 25 mg/kg of diethylnitrosamine (DEN, Sigma) at 15 days of age. Mice were sacrificed 6-8-10-12 months later, to analyze liver cancer. Liver

cancer score was based on number and volume of lesions (0: no lesions; 1: lesion number<3, or lesion dimension <3mm; 2: lesion number<5, or lesion dimension <5mm; 3: lesion number<10, or lesion dimension <10mm; 4: lesion number<15, or lesion dimension <10mm; 5: lesion number>15, or lesion dimension >10mm). Lung metastasis experiments were performed injecting i.m. the 3-MCA derived mycoplasma-free sarcoma cell line MN/MCA1 (10^5 cells/mouse in 100 μ l PBS) [344]. Primary tumor growth was monitored twice weekly, and lung metastases were assessed by *in vivo* imaging and by macroscopic counting at sacrifice 25 days after injection. Liver metastases were generated by injecting intrasplenically 1.5×10^5 mycoplasma-free colon carcinoma cells (MC38) [345]. Mice were sacrificed 12 days after injection and liver metastasis were counted macroscopically. MC38 cells were received from ATCC just before use. MN/MCA1 cells were authenticated morphologically by microscopy *in vitro* and by histology *ex vivo*. Tumor size limit at which mice were sacrificed was based on major diameter (≤ 2 cm).

4.16 Viral infections

Mice were injected intravenously (i.v.) with 5×10^5 PFU of the tissue culture (TC)-grown virus in PBS. Wild-type MCMV or mutant MCMV lacking m157 were used as indicated [346]. Bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 has been previously shown to be biologically equivalent to MCMV strain Smith (VR-1399) and is hereafter referred to as wild-type (WT) MCMV [347]; mutant m157 MCMV is named Δ m157 MCMV. Mice were sacrificed 1.5 and 4.5 days post infection and viral titer was assessed by plaque assay, as previously described [348, 349]. Newborn mice were infected i.p. with 2000 PFU of the MCMV strain MW97.01 and sacrificed at day 7 post infection. Viral titer was assessed by plaque assay, as previously described [348, 349].

4.17 Adoptive transfer

10^6 *Il1r8*^{+/+} or *Il1r8*^{-/-} sorted NK cells were injected i.v. in wild type adult mice 5 hours before MN/MCA or MC38 injection, or i.p. in newborn mice 48 hours after MCMV injection. Adoptively transferred NK cell engraftment, proliferative capacity and functionality (IFN γ production and degranulation after *ex vivo* stimulation) were assessed 3 and 7 days after injection.

4.18 In vivo proliferation

In vivo proliferation was measured using Click-iT[®] Edu Flow Cytometry Assay Kit (Invitrogen). Edu was injected i.p. (0.5 mg/mouse), mice were sacrificed 24 hours later and cells were stained following the manufacturer's instructions and analyzed by flow cytometry.

4.19 Immunohistochemistry

Liver frozen tissues were cut at 8 μ m and then fixed with 4% PFA. Endogenous peroxidases were blocked with 0.03% of H₂O₂ for 5 min and unspecific binding sites were blocked with PBS + 1% FBS for 1h. Tissues were stained with polyclonal goat anti mouse NKp46/NCR1 (R&D System) and goat on mouse HRP polymer kit (GHP516, Biocare Medical) was used as secondary antibody. Reactions were developed with 3,3'-Diaminobenzidine (DAB) (Biocare Medical) and then slides were counterstained with hematoxylin. Slides were mounted with eukitt (Sigma-Aldrich). 20X images were analyzed with cell[^]F software (Olympus).

4.20 *In vivo* Imaging

After feeding with AIN-76A alfalfa-free diet (Mucedola srl, Italy) for two weeks, to reduce fluorescence background, mice were intravenously (i.v.) injected with XenoLight RediJect 2-DeoxyGlucosone (DG) (PerkinElmer) and 24 hours later 2-DG fluorescence was measured using Fluorescence Molecular Tomography system (FMT 2000, Perkin Elmer). Acquired images were subsequently analyzed with TrueQuant 3.1 analysis software (Perkin Elmer).

4.21 Statistical analysis

For animal studies, sample size was defined on the basis of past experience on cancer and infection models, in order to detect differences of 20% or greater between the groups (10% significance level and 80% power). Values were expressed as mean \pm SEM or median of biological replicates, as specified. One-way ANOVA or Kruskal-Wallis test were used to compare multiple groups. Two-sided unpaired Student's *t* test was used to compare unmatched groups with Gaussian distribution and Welch's correction was applied in case of significantly different variance. Mann-Whitney test was used in case of non-Gaussian distribution. ROUT test was applied to exclude outliers. $p \leq 0.05$ was considered significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented. Statistics were calculated with GraphPad Prism version 6, GraphPad Software.

5. Results

5.1 Role of IL-1R8 in NK cell differentiation and function

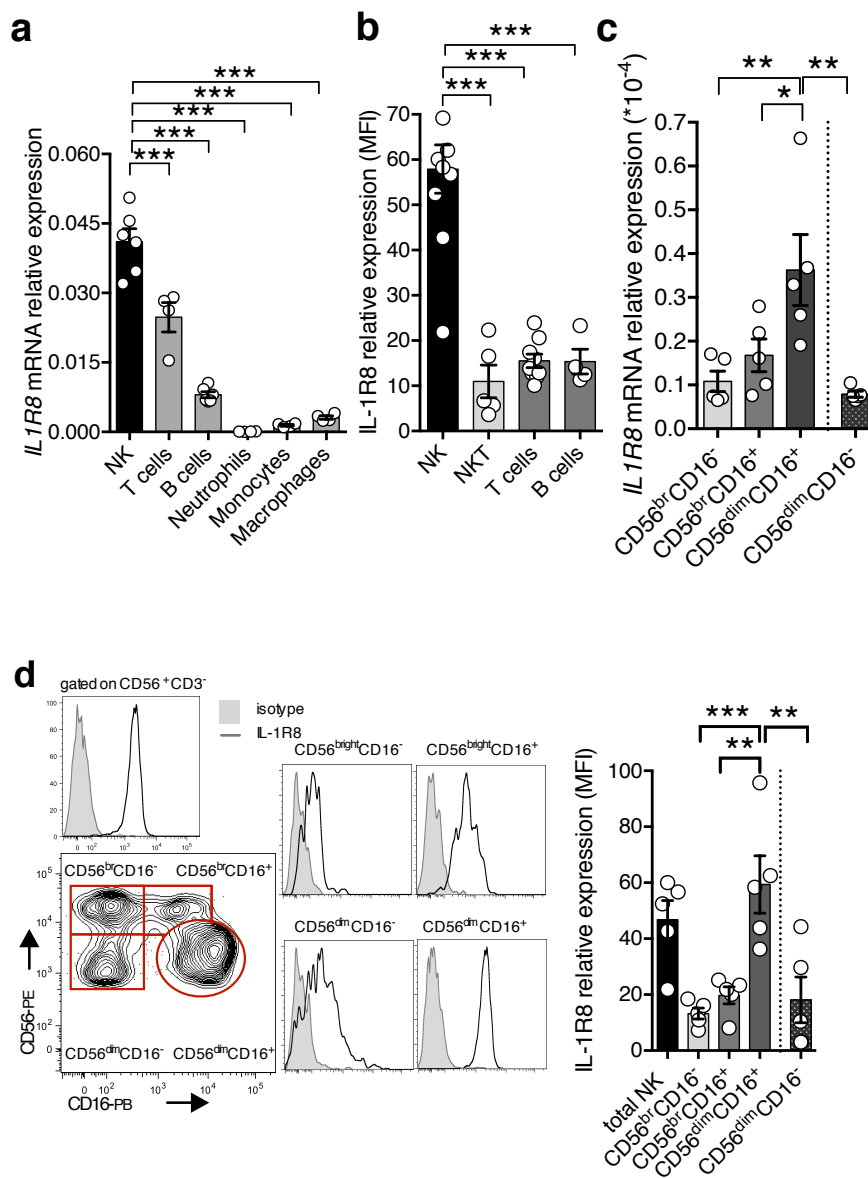
5.1.1 IL-1R8 is differentially expressed in human and murine NK cell subpopulations

IL-1R8 mRNA was originally observed in human primary NK cells by Northern blot analysis [34]. As shown in Figure 1, we confirmed IL-1R8 mRNA and protein expression in human NK cells purified from peripheral blood. IL-1R8 mRNA levels were considerably higher in peripheral blood NK cells, compared to other circulating leukocytes (e.g. T cells, B cells, neutrophils and monocytes) and *in vitro*-derived macrophages (Figure 1A). In agreement, IL-1R8 protein expression level was higher, compared with NKT, T and B cells (Figure 1B). Upon sorting of NK subsets based on CD56 and CD16 expression, we observed that *IL1R8* mRNA level increased during NK cell maturation, and it was higher in the CD56^{dim}CD16⁺ more mature subset compared to the CD56^{br}CD16⁻ subset (Figure 1C). Flow cytometry analysis showed that IL-1R8 expression pattern in each NK cell subset was in line with mRNA transcript levels (Figure 1D). Flow cytometry analysis of human bone marrow cells showed that IL-1R8 expression was acquired in early hematopoietic precursors (e.g. HSC), NK cell-committed precursors (e.g. NKP) and immature NK cells and it was selectively upregulated in mature NK cells, but not in T and NKT cells (Figure 1E).

We next explored IL-1R8 expression on murine NK cells. Murine NK cells expressed high levels of *Il1r8* mRNA, compared with other cell types (CD4⁺ T, CD8⁺ T and B cells,

neutrophils and *in vitro*-derived macrophages) and with other IL-1 family receptors in NK cells (*Il1r1*, *Il1r2*, *Il1r3*, *Il1r4*, *Il1r5* and *Il1r6*) (Figure 1F, 1G). Confocal microscopy analysis showed that murine splenic NK cells express high levels of IL-1R8 cell-membrane protein (Figure 1H). In line with the result obtained in human NK cells, *Il1r8* mRNA level increased during the 4-stage developmental transition from CD11b^{low}CD27^{low} to CD27^{low} (Figure 1I).

These results indicate that both human and murine NK cells express IL-1R8, which is upregulated during NK cell maturation.



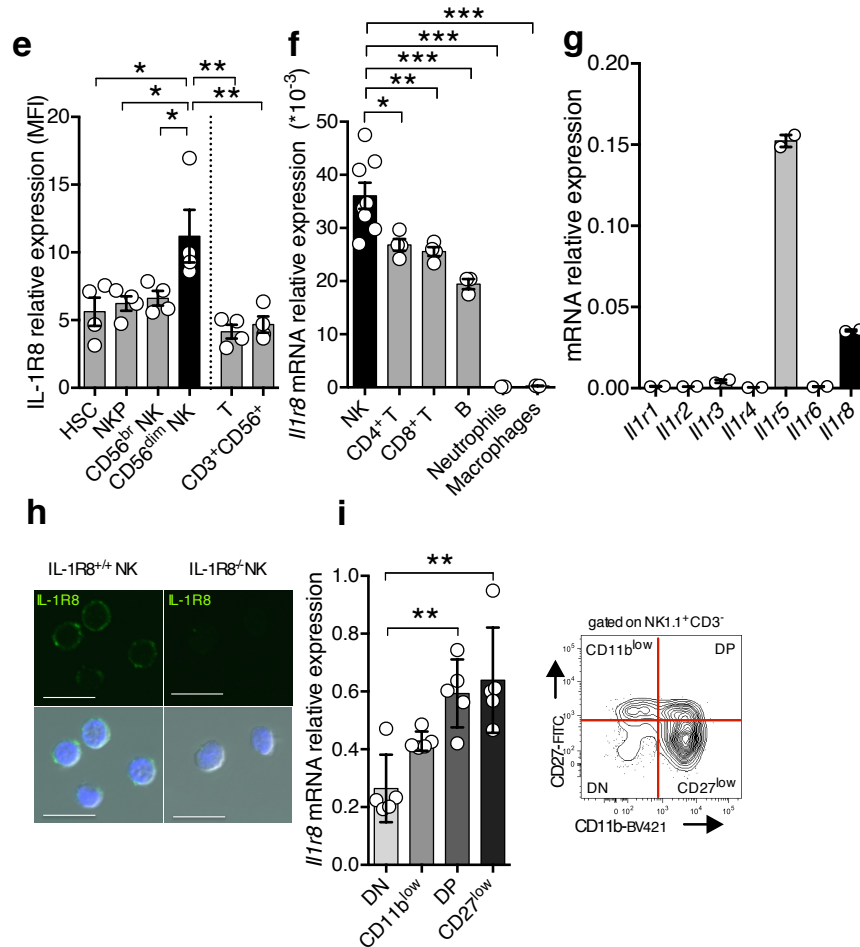


Figure 1. IL-1R8 expression in NK cells. (a, b and c) IL-1R8 mRNA (a, c) and protein (b) expression in human primary NK cells, compared with T and B cells, neutrophils, monocytes and in vitro-derived macrophages (a) or T, B and NKT cells (b) and in human primary NK cell maturation stages (CD56^{br}CD16⁻, CD56^{br}CD16⁺, CD56^{dim}CD16⁺), and in the CD56^{dim}CD16⁺ subset (c). (d) IL-1R8 protein expression in human primary NK cells and NK cell subsets. (e) IL-1R8 protein expression in human bone marrow precursors and mature cells. (f) IL-1R8 mRNA expression in murine primary NK cells, compared with T and B cells, neutrophils, and in vitro-derived macrophages. (g) IL-1 receptor family members (Il1r1, Il1r2, Il1r3, Il1r4, Il1r5, Il1r6, Il1r8) mRNA expression in murine primary NK cells isolated from the spleen. (h) IL-1R8 protein expression in murine NK cells by confocal microscopy. Magnification bar: 10μm. (i) Il1r8 mRNA expression in murine NK cell subsets (DN, CD11b^{low}, DP and CD27^{low}) sorted from splenic NK cells. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mean ± SEM is represented)

5.1.2 IL-1R8-deficient NK cells display a more mature phenotype

In order to address the role of IL-1R8 in NK cells, we analyzed the phenotype of NK cells in *Il1r8*-deficient mice. Both NK cell frequency among lymphocytes and NK cell absolute number were significantly higher in peripheral blood of *Il1r8*^{-/-} compared to *Il1r8*^{+/+} mice. A minor increase was observed in the liver, but not in the spleen of *Il1r8*^{-/-} mice, whereas frequency and absolute number were lower in the BM of *Il1r8*^{-/-} mice (Figure 2A, 2B).

We next analysed the maturation of NK cells in *Il1r8*^{-/-} mice. In mice, most NK cells develop in the bone marrow [108, 141], and their maturation can be dissected/identified based on the expression of CD27, a TNF receptor family member, together with the integrin CD11b. During maturation, NK cells progressively acquire the expression on the cell surface of CD11b, while CD27 is gradually lost, resulting in three subsets: CD11b^{low}CD27^{high} NK cells, CD11b^{high}CD27^{high} NK cells and CD11b^{high}CD27^{low} NK cells [131]. In *Il1r8*^{-/-} mice, the frequency of the CD27^{low} subset was significantly higher compared to *Il1r8*^{+/+} mice (p<0.001) in BM, spleen and blood, indicating a more mature phenotype of NK cells (Figure 2C). The increased frequency of the CD27^{low} subset was counterbalanced by a reduction of the CD11b^{high}CD27^{high} and CD11b^{low}CD27^{high} subsets in *Il1r8*^{-/-} mice. *Il1r8*^{-/-} NK cells collected from BM, spleen and blood expressed higher levels of the maturation marker KLRG1 (Figure 2D). Increased expression of KLRG1 was not associated to a specific NK subset (CD11b^{low}CD27^{high}, CD11b^{high}CD27^{high} and CD11b^{high}CD27^{low}) (Figure 2E), suggesting that the higher frequency of KLRG1⁺ NK cells in the whole NK cell population is due to higher frequency of CD11b^{high}CD27^{low} NK cells. Finally, we analysed Ly49 receptors, which are clonally acquired during NK cell differentiation and we did not observed any difference in *Il1r8*^{-/-} NK cells, compared to *Il1r8*^{+/+} NK cells (Figure 2F). This suggests that

the NK cell education process is not altered in IL-1R8-deficient mice and IL-1R8 does not affect the MHCI-mediated licensing of NK cells.

Since NK cell maturation occurs in the first 2-3 weeks of age [131], we investigated whether the more mature phenotype of NK cells occurred early during development of *Il1r8*^{-/-} mice. As shown in Figure 3A and 3B, the frequency of CD27^{low} and DP subsets was higher in *Il1r8*^{-/-} mice already at 2 and 3 weeks of age, indicating an early acquisition of a more mature phenotype in NK cells in newborn mice. Moreover, at 3 weeks of age, the number of total NK cells was higher in spleen and blood in *Il1r8*^{-/-} mice (Figure 3A, 3B). We next addressed whether IL-1R8-deficiency affected the development of NK precursors in BM. As shown in Figure 3C, the frequency of hematopoietic stem cells (HSC), common lymphoid precursors (CLP), pre-NK precursors (pre-NKP) and NK precursors (NKP) was similar in *Il1r8*^{-/-} and *Il1r8*^{+/+} mice. Collectively, these results suggest that IL-1R8 is involved in the regulation of NK cell maturation but does not affect NK cell commitment, and in absence of IL-1R8 NK cells display a more mature phenotype, which is early acquired in newborn mice.

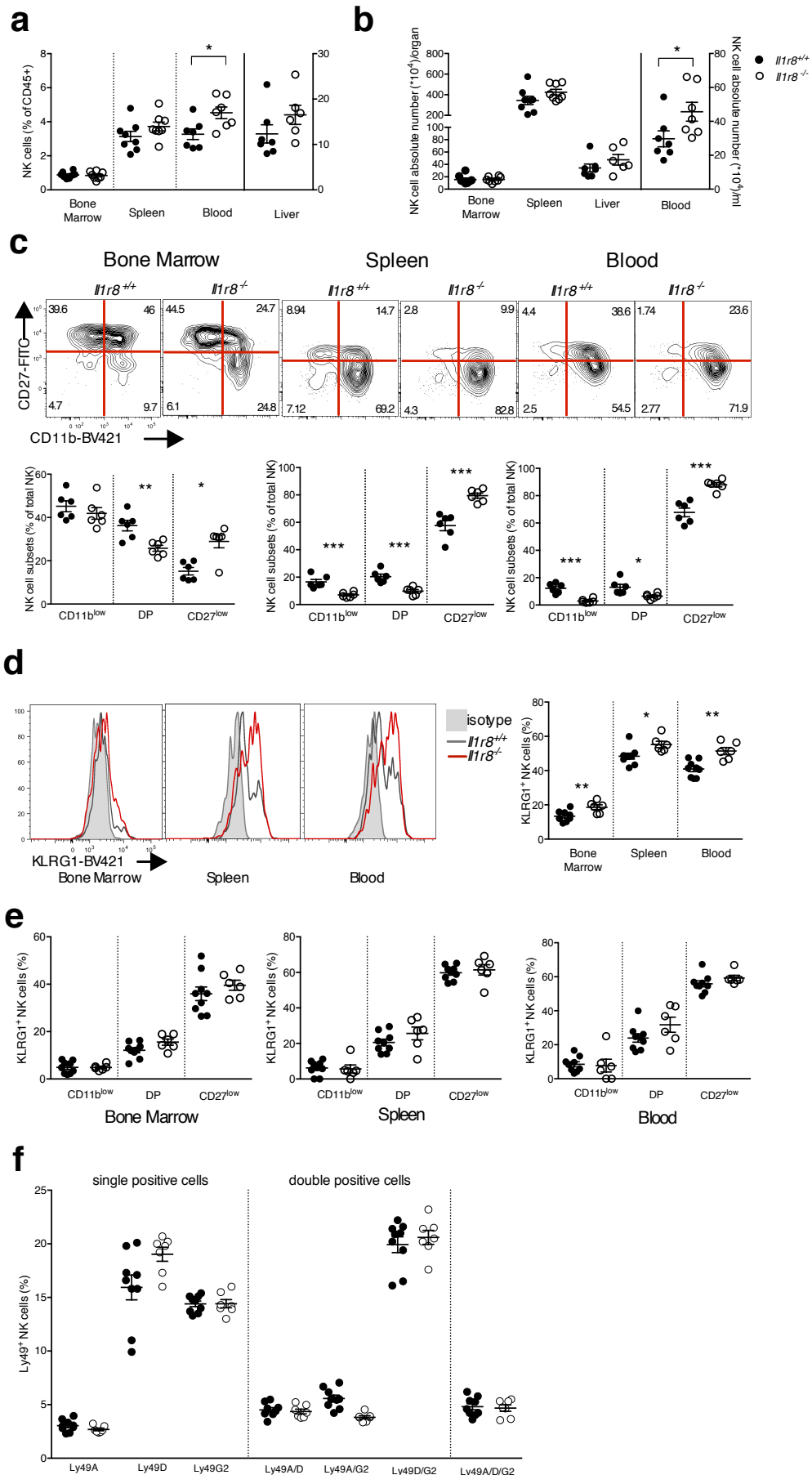


Figure 2. NK cell differentiation in IL-1R8-deficient mice. (a, b) NK cell frequency and absolute number among leukocytes in bone marrow, spleen, blood and liver of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice. (c) NK cell subsets (DN, $CD11b^{low}$, DP and $CD27^{low}$) in bone marrow, spleen and blood of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice. (d, e) KLRG1 expression in NK cells in bone marrow, spleen and blood of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice, in total NK cells (d) and NK cell subsets (e). (f) Frequency of Ly49A, Ly49D and Ly49G single positive, double positive and triple positive NK cells in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

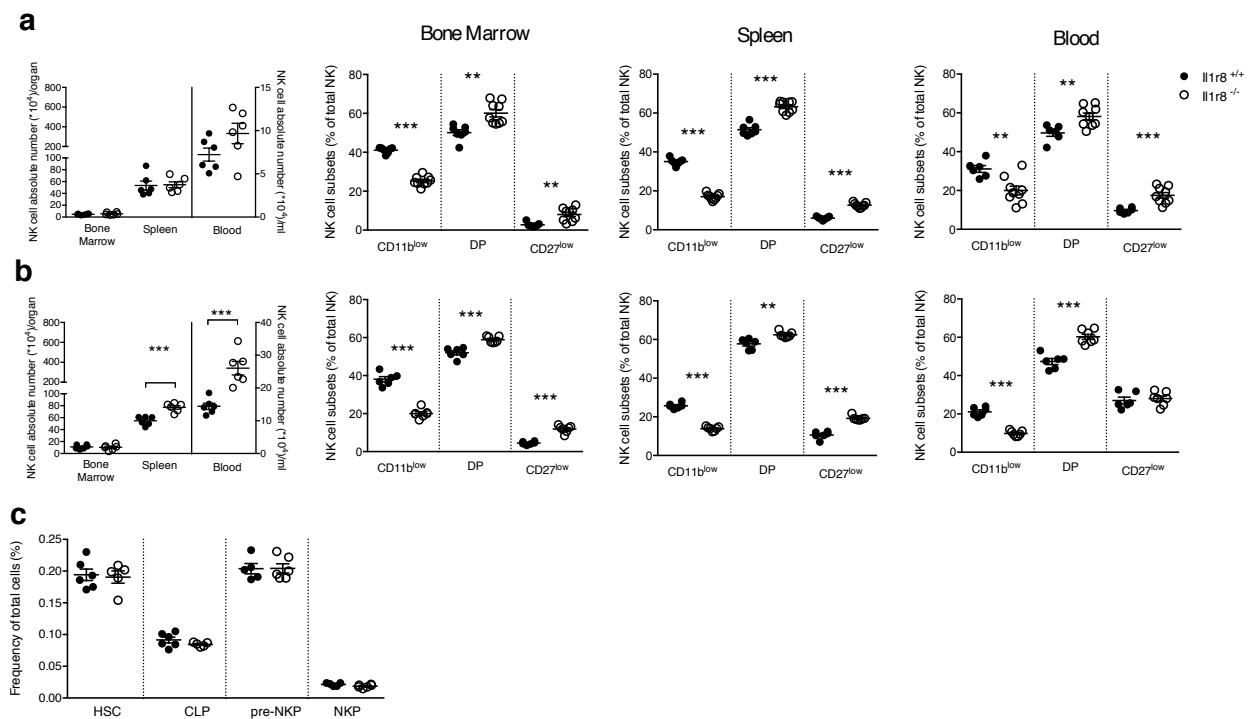


Figure 3. Early stages of NK cell maturation. (a, b) NK absolute number and NK cell subsets (DN, $CD11b^{low}$, DP and $CD27^{low}$) in bone marrow, spleen and blood of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ newborn mice at 2 (a) and 3 (b) weeks of age. (c) Frequency of bone marrow precursors (HSC, CLP, pre-NKP, NKP) in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

5.1.3 IL-1R8-deficient NK cells express higher levels of activating receptors and display enhanced effector functions

We next investigated whether IL-1R8 impacted on NK cell activities. We first analysed the expression of NKG2D and DNAM-1, two activating receptors involved in the recognition of tumor cells and in NK cell cytotoxicity. NKG2D expression was significantly upregulated in peripheral blood *Il1r8*^{-/-} NK cells, in particular in CD11b^{low}CD27^{high} and CD11b^{high}CD27^{high} subsets (Figure 4A). DNAM-1 expression was significantly increased only in CD11b^{high}CD27^{high} NK cells (Figure 4B). We then characterized the expression of Ly49H, an activating receptor that specifically recognizes the MCMV encoded-protein m157. Higher levels of Ly49H were observed in *Il1r8*^{-/-} NK cells, in particular in the CD11b^{high}CD27^{low} subset (Figure 4C).

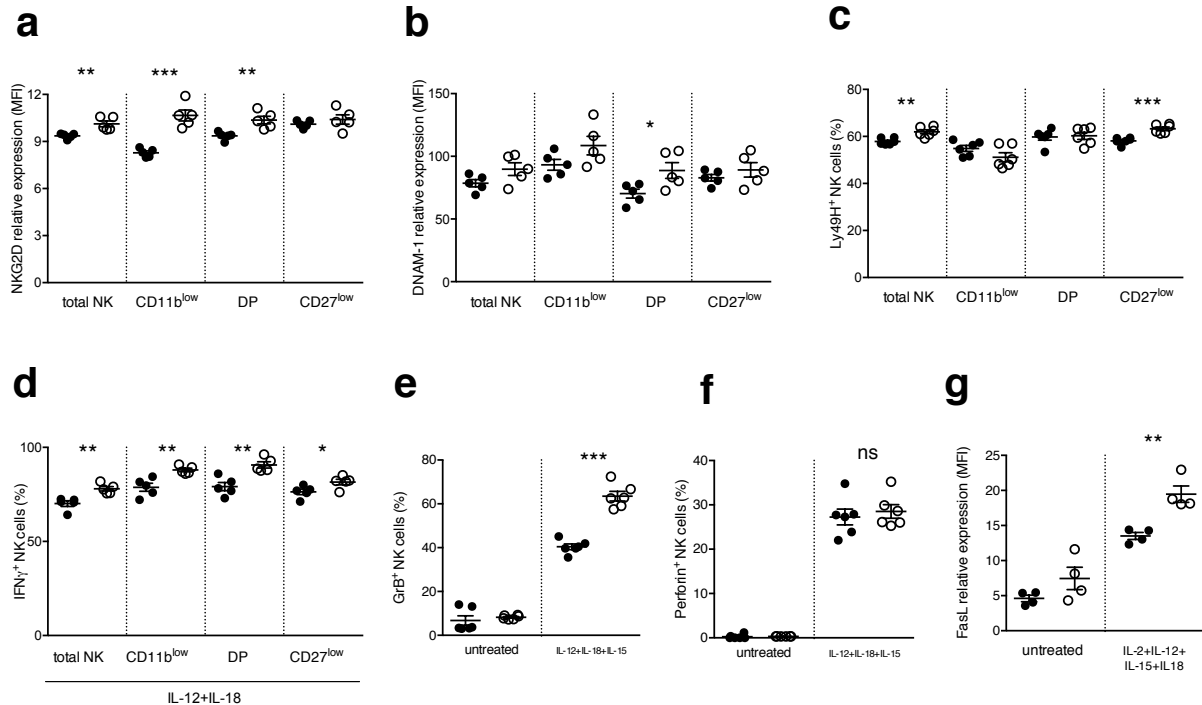


Figure 4. IL-1R8-deficient NK cell effector functions. (a-c) NKG2D (a), DNAM-1 (b) and LY49H (c) expression in peripheral NK cells and NK cell subsets of *Il1r8*^{+/+} and *Il1r8*^{-/-} mice. (d-g) IFN γ (d), Granzyme B

(e), perforin (f) and FasL (g) expression in *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells and NK cell subsets upon ex-vivo stimulation. (*p < 0.05, **p < 0.01, ***p < 0.001, Mean ± SEM is represented)

To establish whether IL-1R8 regulates NK cell effector functions, we evaluated IFN γ production and degranulation in *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells, upon ex-vivo stimulation with a combination of IL-12 and IL-18. As shown in Figure 4D, IL-12/IL-18 stimulation drove a more sustained IFN- γ response in IL-1R8-deficient NK cells. The frequency of IFN- γ ⁺ NK cells was higher in *Il1r8*^{-/-} total NK cells and in each NK cell subset (CD11b^{high}CD27^{low}, CD11b^{high}CD27^{high} and CD11b^{low}CD27^{high}). This indicates that IFN- γ production was enhanced by the absence of IL-1R8 in all NK cells, independently from their maturation state. We then analysed the expression of cytotoxic mediators in *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells, upon treatment with IL-12, IL-15 and IL-18. As shown in Figure 4E and 4F, the percentage of granzyme B⁺ NK cells was higher in absence of IL-1R8, whereas no difference in terms of perforin production was observed. Since IL-18 is also involved in the expression of FasL and activation of FasL-mediated killing by NK cells (Smyth MJ 2004), we assessed whether IL-1R8 might regulate FasL expression. Indeed, *Il1r8*^{-/-} NK cells expressed higher levels of FasL, upon stimulation with a combination of IL-2, IL-12, IL-15 and IL-18, suggesting that IL-1R8 could also negatively regulate FasL-dependent effector mechanisms in NK cells (Figure 4G). Overall, these results showed that IL-1R8 negatively regulates NK cell effector functions, suggesting that in absence of IL-1R8 NK cell activation threshold is lowered.

5.1.4 IL-1R8 directly regulates NK cell differentiation through a cell-autonomous mechanism

In addition to leukocytes, IL-1R8 is expressed by different cell types, including epithelial cells. To characterize the mechanism responsible for the more sustained NK cell differentiation and activation observed in the absence of IL-1R8 and dissect the contribution of the hematopoietic and non-hematopoietic compartments, we first generated bone-marrow chimeric mice. *Il1r8*^{+/+} or *Il1r8*^{-/-} bone marrow cells were transplanted into either *Il1r8*^{+/+} or *Il1r8*^{-/-} recipients, and the phenotype of NK cells in these mice were analysed 9 weeks upon transplantation. As shown in Figure 5A and 5B, both absolute numbers and maturation stages of NK cells were not affected by the host genotype. Indeed, *Il1r8*^{+/+} NK cells developed in *Il1r8*^{-/-} hosts had a phenotype overlapping that of *Il1r8*^{+/+} NK cells developed in *Il1r8*^{+/+} host and in turn, *Il1r8*^{-/-} NK cells developed in *Il1r8*^{+/+} hosts resembled *Il1r8*^{-/-} NK cells developed in *Il1r8*^{-/-} hosts. Thus, IL-1R8-deficient BM drove the generation of a higher absolute number of NK cells in the periphery and these cells displayed a more mature phenotype, suggesting that IL-1R8-mediated regulation in NK cells is attributable to the hematopoietic compartment. To understand whether the regulatory function of IL-1R8 was NK cell-intrinsic or it was dependent on the contribution of other leukocytes, we generated competitive bone-marrow chimeric mice transplanting a mix of *Il1r8*^{+/+} CD45.1 and *Il1r8*^{-/-} CD45.2 BM cell in both *Il1r8*^{+/+} and *Il1r8*^{-/-} hosts. *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells in these mice differentiated in the same competitive environment and their maturation was consequently driven by the same stimuli. Upon reconstitution, the frequency of *Il1r8*^{-/-} NK cells was higher in the periphery and their phenotype was more mature, compared with those of *Il1r8*^{+/+} NK cells, in both *Il1r8*^{+/+} and *Il1r8*^{-/-} recipients (Figure 5C and 5D).

Along the same line, co-culture experiments of NK cells with LPS- or CpG-primed dendritic

cells (DCs) showed that *Il1r8*^{-/-} NK cells produced higher IFN γ levels irrespective of the DC genotype (Figure 5E, 5F).

These results demonstrated that the negative regulation exerted by IL-1R8 was an intrinsic mechanism in NK cells and excluded that IL-1R8-deficiency in non-hematopoietic cell types or in other leukocytes could lead to the generation of a microenvironment that promoted NK cell differentiation.

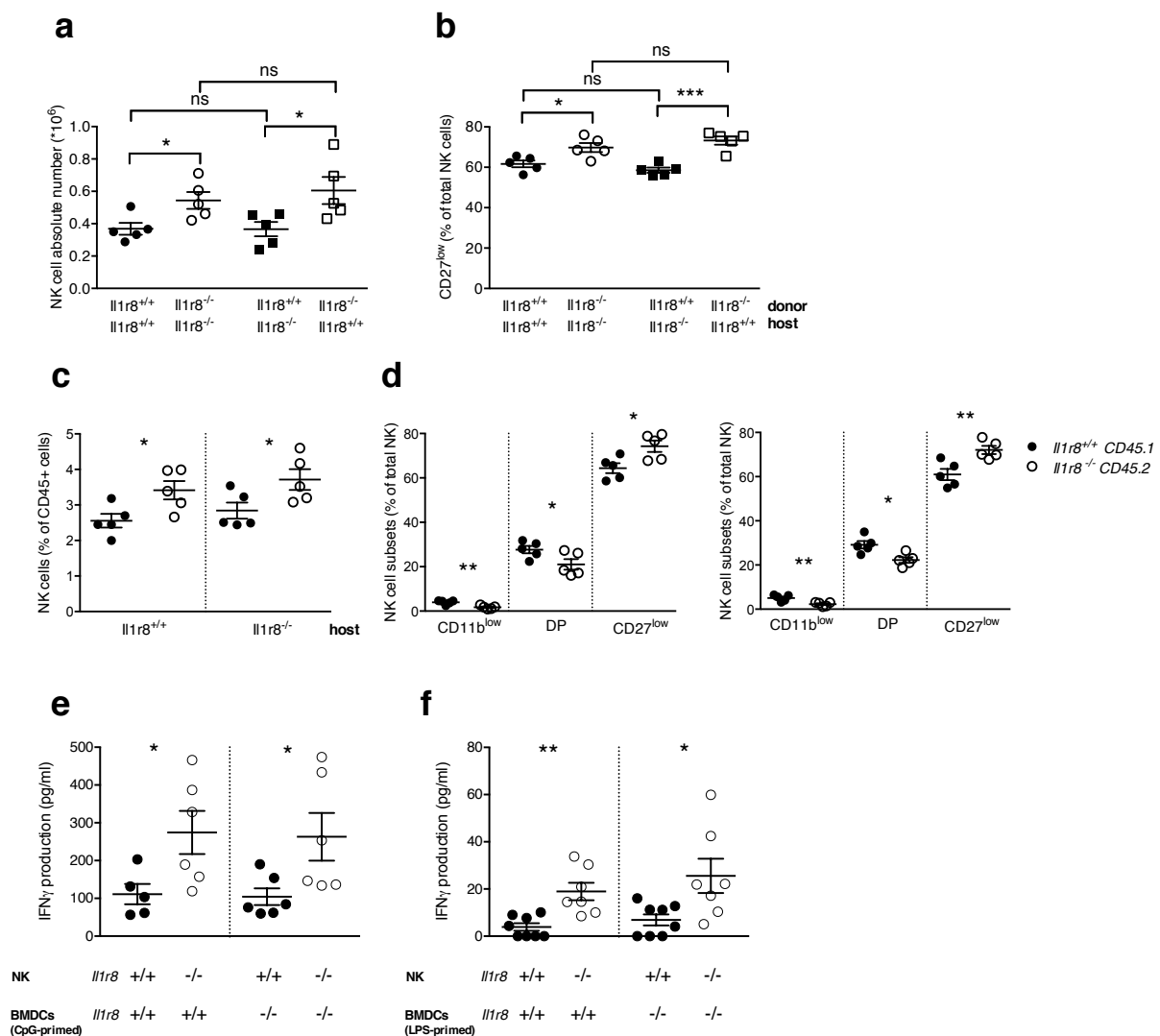


Figure 5. The enhanced maturation and activation of *Il1r8*^{-/-} NK cell is a cell autonomous mechanism.

(a and b) Peripheral NK cell absolute number (a) and CD27^{low} NK cell frequency (b) in bone marrow chimeric mice upon reconstitution (9 weeks). (c and d) Peripheral NK cell (c) and NK cell subset (d)

frequency in competitive chimeric mice transplanted with 50% of *Il1r8*^{+/+} CD45.1 cells and 50% of *Il1r8*^{-/-} CD45.2 cells upon reconstitution (9 weeks). Upon reconstitution a defective engraftment (12% instead of 50% engraftment) of *Il1r8*^{-/-} stem cells was observed in competitive conditions. (e, f) IFN γ production by *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells upon co-culture with LPS- or CpG-primed *Il1r8*^{+/+} and *Il1r8*^{-/-} DCs. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mean \pm SEM is represented)

5.1.5 IL-1R8 regulates NK cell differentiation through the inhibition of IL-18 pathway

The enhanced differentiation of *Il1r8*^{-/-} NK cells suggested hyper-activation of an IL-1R8-targeted pathway in this cell type. To dissect the pathway involved, we first considered the contribution of MyD88, which is a key transcription factor downstream of any IL-1R8-targeted molecule. MyD88-deficiency led to a reduced frequency of mature NK cells in the spleen, measured as CD11b⁺ NK cell frequency (Figure 6A). Since IL-18 is one of the major cytokines involved in NK cell differentiation and function and is targeted by IL-1R8, we next evaluated the role of IL-18 in *Il1r8*^{-/-} NK cell phenotype. As shown in Figure 6B, the depletion of IL-18 *in vivo* with an anti-IL-18 neutralizing antibody rescued the effect of IL-1R8-deficiency on NK cell differentiation. Indeed, upon IL-18 neutralization, no difference was observed in the frequency of CD27^{low} NK cells between *Il1r8*^{+/+} and *Il1r8*^{-/-} mice. Treatment with a control isotype antibody did not affect the enhanced differentiation of NK cells in *Il1r8*^{-/-} mice, compared with *Il1r8*^{+/+} mice. In agreement, IL-18 genetic blockade also abolished the phenotype observed in *Il1r8*^{-/-} mice (Figure 6C). Thus, IL-1R8 suppressed IL-18-mediated stimulation of NK cells and consequently inhibited NK cell maturation.

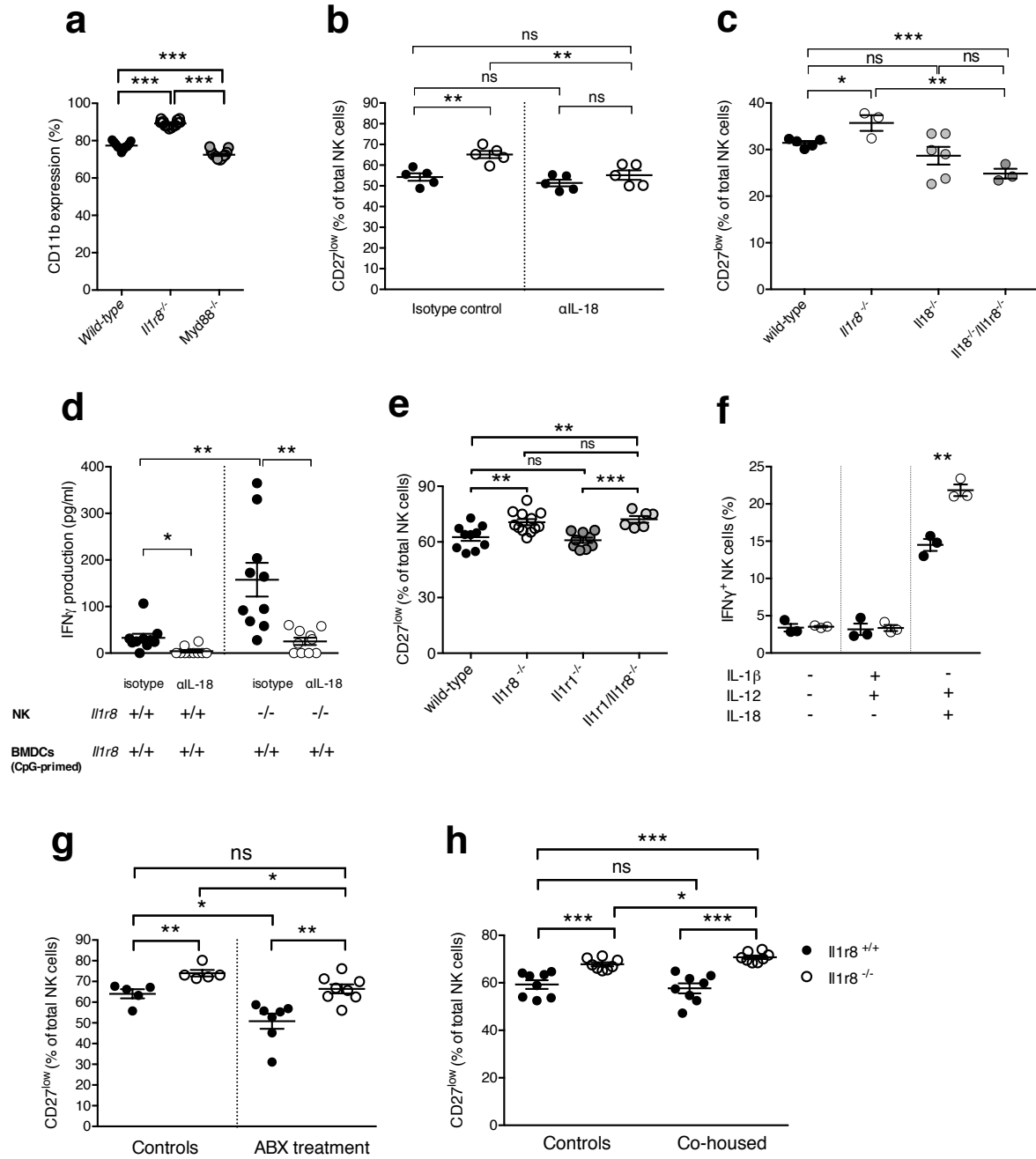


Figure 6. IL-1R8-deficient NK cell phenotype is dependent on IL-18. (a) NK cell maturation in *Myd88*-deficient mice. (b, c) Splenic CD27^{low} NK cell frequency in *Il1r8*^{+/+} and *Il1r8*^{-/-} mice upon IL-18 in vivo depletion (b) or in *Il18*^{-/-}/*Il1r8*^{-/-} (c). (d) IFN γ production by *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells upon co-culture with CpG-primed *Il1r8*^{+/+} DCs and IL-18 blockade. (e, f) Peripheral CD27^{low} NK cell frequency in wild-type, *Il1r8*^{-/-}, *Il1r1*^{-/-} and *Il1r8*^{-/-}/*Il1r1*^{-/-} mice (e) and IFN γ production by splenic NK cells after IL-12 and IL-1 β or IL-18 stimulation (f). (g, h) Splenic CD27^{low} NK cell frequency in *Il1r8*^{+/+} and *Il1r8*^{-/-} mice upon commensal flora depletion (g) and breeding in co-housing conditions (h). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

Moreover, IL-18 neutralization rescued the enhanced IFN γ production observed in IL-1R8-deficient NK cells, upon co-culture with CPG-primed DCs, indicating that IL-18 pathway is the major target of IL-1R8-mediated regulation of NK cell effector functions (Figure 6D).

To exclude the involvement of other ILRs and TLRs in the IL-1R8-dependent regulation of NK cells, we evaluated the role of IL-1R1 signalling and the contribution of the commensal microflora in NK cell differentiation and function, in *Il1r8*^{-/-} mice. *Il1r1*^{-/-} mice displayed the same NK cell maturation phenotype as wild-type mice and *Il1r1*^{-/-}/*Il1r8*^{-/-} mice mimicked the phenotype of *Il1r8*^{-/-} mice, suggesting that IL-1R1 was not one of the IL-1R8 targets in this context (Figure 6E). Concerning IL-1-involvement in NK cell activation, we did not observed any NK cell activation in terms of IFN γ production, upon stimulation with IL-12 and IL-1 β , thus excluding IL-1 pathway as a possible target of IL-1R8 in the modulation of NK cell maturation and function (Figure 6F).

Since commensal bacteria are involved in NK cell priming [350], we tested whether IL-1R8 targeted microbiota-derived signals in NK cells, thus affecting NK cell differentiation. Microflora depletion was obtained through oral treatment with antibiotics for 5 weeks and confirmed by 16S rRNA qPCR in fecal pellets (data not shown). In agreement with previous reports, antibiotic treatment impaired the frequency of the CD27^{low} mature NK subset in both *Il1r8*^{+/+} and *Il1r8*^{-/-} mice. However, antibiotic-treatment did not abolish the difference in NK cell maturation between *Il1r8*^{-/-} and *Il1r8*^{+/+} mice. This suggests that the regulation of NK cell differentiation mediated by IL-1R8 is not dependent on commensal microbiota-derived signals, which act through a different mechanism (Figure 6G). Finally, the phenotype of NK cells was not modified by co-housing of *Il1r8*^{+/+} and *Il1r8*^{-/-} mice (Figure 6H), in line with the irrelevance of commensal flora (Figure 6G) and microenvironment (Figure 5A, B, C, D, E, F).

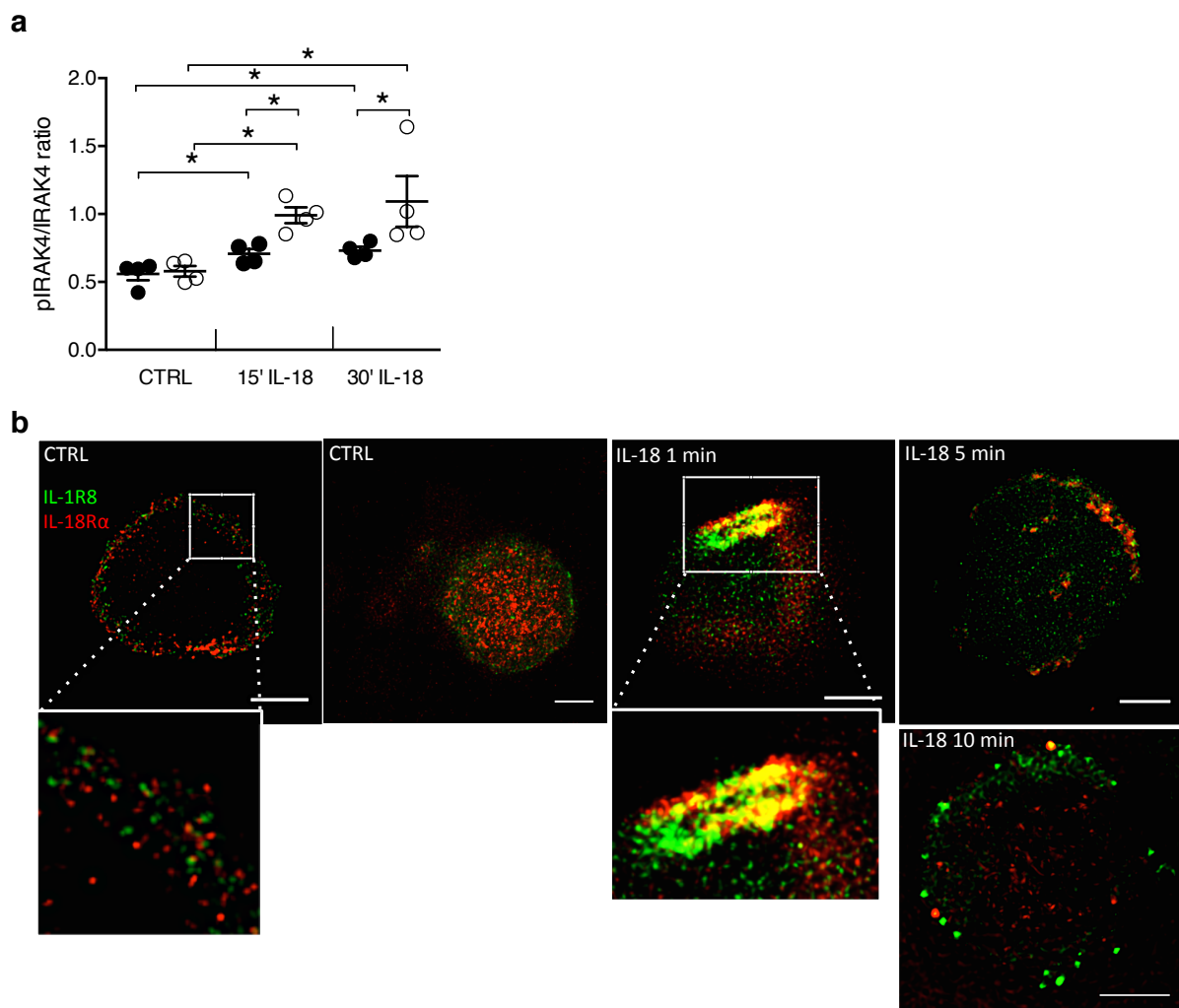
5.1.6 IL-1R8 directly inhibits IL-18-dependent pathways in NK cells

The results reported above show that IL-1R8 regulated IL-18 signalling pathway in NK cells, thus controlling NK cell maturation and function. Indeed, increased phospho-IRAK4/IRAK4 ratio was induced by IL-18 in *Il1r8^{-/-}* NK cells compared to wild type NK cells, indicating unleashed early signaling events downstream of MyD88 and therefore myddosome formation (Figure 7A). This result is consistent with previous reports regarding the molecular mechanism of IL-1R8 activity, including structural analysis. By stimulated emission depletion (STED) microscopy, we observed clustering of IL-1R8 and IL-18R α (Figure 7B), suggesting an interaction between the two molecules as indicated by previous studies.

Since mTOR pathway controls NK cell metabolism and differentiation [351] and it is targeted by IL-1R8 in T cells, we analysed the phosphorylation of key molecules downstream of mTOR activation (e.g. the translation-initiation factor eIF4E-binding protein 4EBP1 and the S6 ribosomal kinase S6K), upon stimulation with IL-18. Given that MAPK/JNK pathway is involved in NK cell activation and it is regulated by IL-1R8, we also considered the phosphorylation of JNK, in response to IL-18 treatment. As shown in Figure 7C, IL-1R8 deficiency led to enhanced phosphorylation of S6 and JNK in splenic NK cells, upon 15' of treatment with IL-18. After 30' of IL-18 stimulation, increased S6 phosphorylation was still observed in *Il1r8^{-/-}* NK cells, whereas JNK phosphorylation returned to the basal level of *Il1r8^{+/+}* controls, indicating a different kinetics of activation of the two pathways. Collectively, these results demonstrated that IL-1R8 inhibited IL-18-dependent activation of mTOR and JNK pathways in NK cells and therefore modulated NK cell maturation.

To obtain a comprehensive picture of the impact of IL-1R8 deficiency on NK cell function and in particular on the response to IL-18, we performed RNA-seq analysis. The bioinformatic analysis of such a big data needed to be careful addressed and we decided to

use the Metascope software to get unbiased information of the differentially expressed genes and thus enriched pathways in IL-1R8-deficient NK cells. As shown in Figure 8A, IL-1R8 deficiency had a profound impact on the transcriptional profile of NK cells, both at steady state conditions and upon stimulation with IL-18. In particular, the enriched pathways observed in IL-18-stimulated NK cells in absence of IL-1R8 included NK cell responsiveness, cytotoxicity, cytokine production and MAPK activation (Figure 8B).



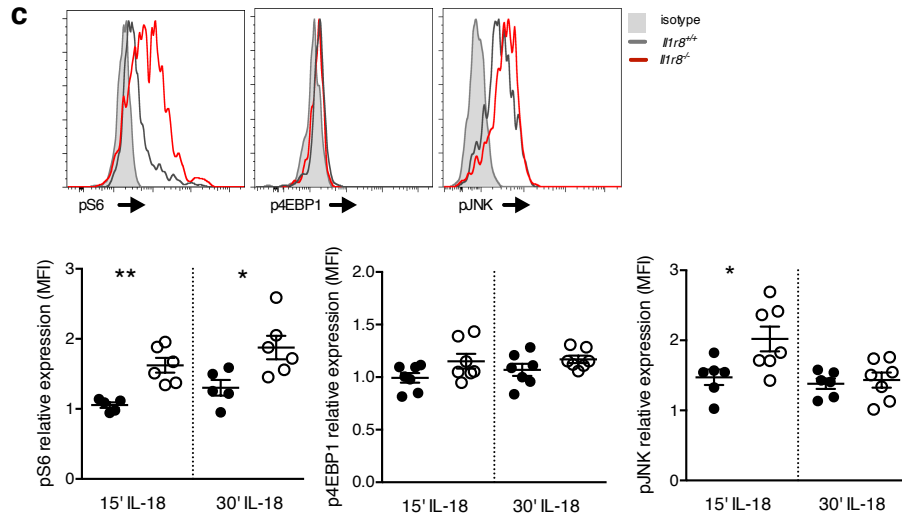


Figure 7. IL-1R8-mediated regulation of IL-18 pathway. (a) IRAK4 phosphorylation rate in *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells upon stimulation with IL-18. (b) STED microscopy of human NK cells stimulated with IL-18. Magnification bar: 2 μ m. (c) S6, 4EBP1 and JNK phosphorylation in *Il1r8*^{+/+} and *Il1r8*^{-/-} NK cells upon stimulation with IL-18. (* p < 0.05, ** p < 0.01, *** p < 0.001, Mean \pm SEM is represented)

to obtain indications that in human NK cells IL-1R8 acts as a checkpoint for activation. First, we retrospectively analyzed IL-1R8 expression in relation to responsiveness to a combination of IL-18 and IL-12 in a series of normal donors. As shown in Figure 9A, we observed an inverse correlation between IL-1R8 levels and IFN γ production in peripheral blood NK cells ($r^2=0.7969$, $p=0.0012$). To assess whether the functional role of IL-1R8 was conserved in human NK cells, we have tried to transfect human primary cells and human NK cell lines (e.g. NKL and NK-92) with several RNA- and DNA-based constructs to silence IL-1R8. We failed to transfect human NK cells by using commonly used transfecting system. In particular, we tested siRNA, Stealth RNAi™ siRNA (Invitrogen) and a CRISPR-Cas9 system, transfecting with Lipofectamin, Lipofectamine® RNAiMAX (Invitrogen) or Amaxa® Nucleofector® Technology (Lonza). We have also tried using a Lentiviral infection system, but the vitality of cells and the infection efficiency were too low. Finally, we succeeded in peripheral blood NK cell transfection using siRNA delivered with the Accell™ medium (GE Healthcare). IL-1R8 partial silencing was associated with a significant increase of CD69 expression and IFN γ production (Figure 9B). These results suggest that in human NK cells as in murine counterparts IL-1R8 works as a negative regulator of activation and that its inactivation unleashes human NK cell effector function.

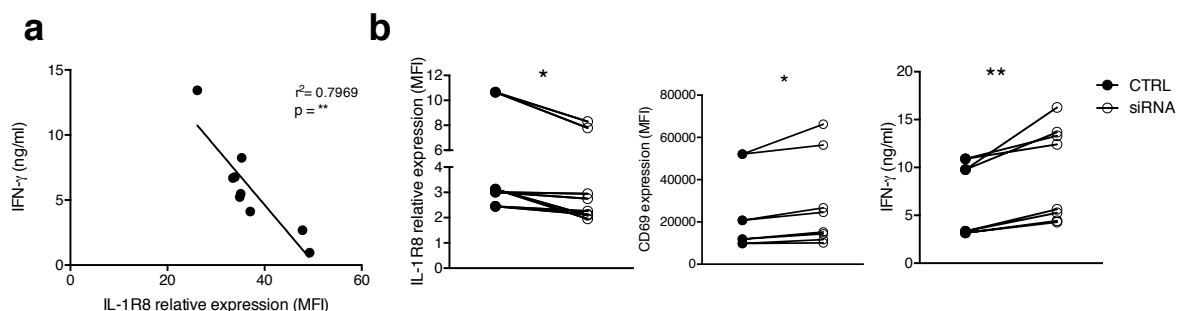


Figure 9. IL-1R8-mediated regulation of human NK cells. (a) Correlation between IL-1R8 expression and IFN γ production in human peripheral blood NK cells. The analysis was conducted retrospectively in

all 9 normal donors tested for response to IL-18 and IL-12. (b) IL-1R8 and CD69 expression and IFN γ production in human NK cells 7 days after transfection with control siRNA or IL-1R8-specific siRNA in duplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

5.2 Relevance in cancer and metastasis

5.2.1 IL-1R8 inhibits NK cell anti-tumor potential in liver cancer

To address the *in vivo* relevance of IL-1R8-dependent negative regulation of NK cell effector functions, we examined anti-cancer and anti-viral resistance. Since the liver is characterized by a high frequency of NK cells [149], we first focused on liver carcinogenesis. We therefore addressed whether IL-1R8-deficiency could favour NK cell activity in a model of Diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) and we analysed the susceptibility of *Il1r8*^{-/-} mice in DEN-induced HCC. IL-1R8-deficient female and male mice were protected against the development of HCC, in terms of macroscopic number and size of lesions (Figure 10A, 10B). The liver macroscopic score was lower in female mice, in agreement with estrogen-dependent gender disparity in this model [352]. The protection against the development of HCC in the absence of IL-1R8 was confirmed by histological analysis, showing significantly higher number of dysplastic ($p=0.02$) and adenoma ($p=0.005$) lesions in 10 month-old HCC-bearing wild type males compared to IL-1R8-deficient hosts (Table 1). The analysis of leukocyte infiltrate by flow cytometry and immunohistochemistry showed that the percentage of NK cells among liver CD45⁺ cells and the absolute number were higher in *Il1r8*^{-/-} HCC-bearing mice (Figure 10C, 10D). In addition, the percentage of IFN- γ ⁺ NK cells was higher in the liver, but not in the spleen of *Il1r8*^{-/-} mice (Figure 10E).

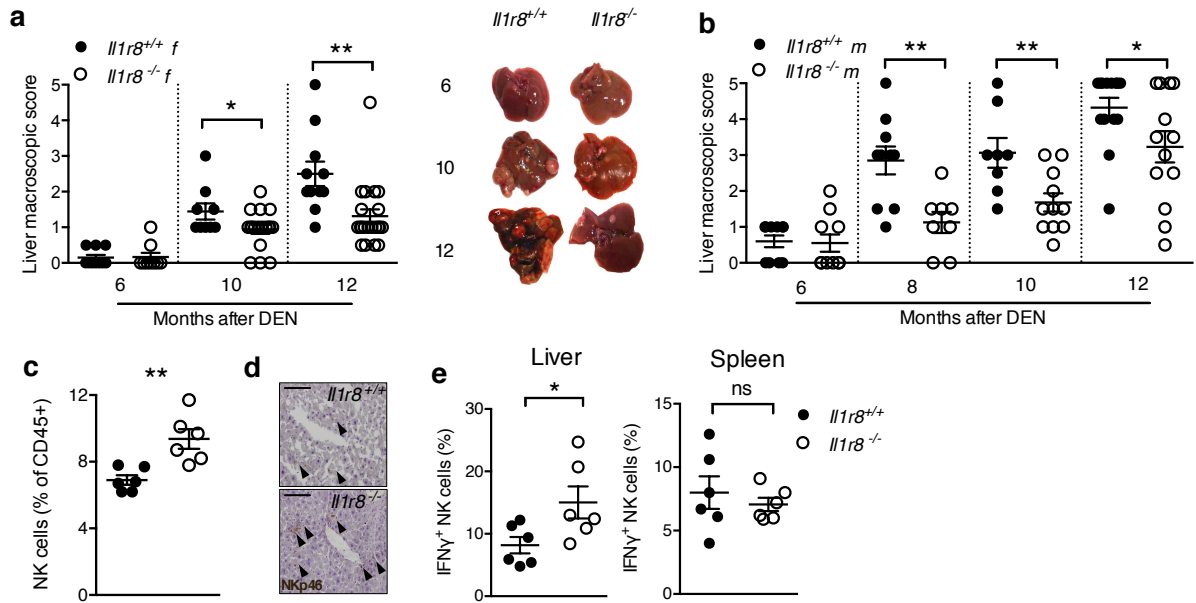


Figure 10. Phenotype of DEN-induced HCC in IL-1R8-deficient mice. (a, b) Macroscopic score of liver lesions in female (a) and male (b) *Il1r8*^{+/+} and *Il1r8*^{-/-} mice 6, 8, 10 and 12 months after DEN injection. (c, d) Frequency (c) and representative histological quantification (d) of NK cell infiltrate in livers of *Il1r8*^{+/+} and *Il1r8*^{-/-} tumor bearing mice (20X, bar = 100μm). (e) Frequency of IFNγ⁺ NK cells in liver and spleen of *Il1r8*^{+/+} and *Il1r8*^{-/-} tumor bearing mice. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mean ± SEM is represented)

T cell subsets were not appreciably altered in normal and tumor bearing *Il1r8*^{-/-} mice (not shown). Finally, increased levels of cytokines involved in anti-tumor immunity (e.g. IFNγ) and a reduction of pro-inflammatory cytokines associated with tumor promotion (IL-6, TNFα, IL-1β, CCL2, CXCL1) were observed (Table 2). Importantly, to address the direct role of IL-1R8-deficient NK cells in the protection, we depleted NK cells in *Il1r8*^{+/+} and *Il1r8*^{-/-} DEN-treated mice using an anti-NK1.1 neutralizing antibody. The depletion of NK cells abolished the protection against liver carcinogenesis observed in both male and female *Il1r8*^{-/-} mice (Figure 11A, 11B), demonstrating that IL-1R8 deficiency enhanced NK cell antitumor potential in the model of DEN-induced HCC.

	8 Months			10 Months			12 Months		
	Number of lesions, mean \pm (SEM)			Number of lesions, mean \pm (SEM)			Number of lesions, mean \pm (SEM)		
Lesion	<i>Il1r8</i> ^{+/+}	<i>Il1r8</i> ^{-/-}	P	<i>Il1r8</i> ^{+/+}	<i>Il1r8</i> ^{-/-}	P	<i>Il1r8</i> ^{+/+}	<i>Il1r8</i> ^{-/-}	P
Displasia	5.4 \pm (1.3)	3.6 \pm (1.4)	0.26	7.8 \pm (1.0)	6.0 \pm (1.4)	0.02	6.3 \pm (0.8)	3.4 \pm (0.8)	0.03
Adenoma	1.3 \pm (0.49)	0.37 \pm (0.1)	0.3	3.2 \pm (0.7)	0.9 \pm (0.3)	0.005	3.0 \pm (0.7)	1.0 \pm (0.2)	0.06
Carcinoma	0.0 \pm (0.0)	0.0 \pm (0.0)	nd	0.0 \pm (0.0)	0.07 \pm (0.0)	nd	0.12 \pm (0.0)	0.1 \pm (0.1)	nd

Table 1. Histological analysis of HCC-bearing mice.

Number of lesions in *Il1r8*^{+/+} and *Il1r8*^{-/-} HCC-bearing mice is listed.

Cytokine pg/mL	6 months after DEN			8-10 months after DEN			12 months after DEN		
	<i>Il1r8</i> ^{+/+} n=4-5*	<i>Il1r8</i> ^{-/-} n=5	p value	<i>Il1r8</i> ^{+/+} n=7-10*	<i>Il1r8</i> ^{-/-} n=9-10*	p value	<i>Il1r8</i> ^{+/+} n=3-5*	<i>Il1r8</i> ^{-/-} n=3-5*	p value
IL-23	173.1 \pm 29.12	247.3 \pm 15.16	0.05	187.7 \pm 13.47	343.4 \pm 66.29	0.04	103.7 \pm 26.72	138.6 \pm 37.51	0.47
IL-12p70	277.6 \pm 44.49	358.4 \pm 12.44	0.12	293 \pm 16.31	357.2 \pm 34.77	0.13	152 \pm 20.14	164.9 \pm 15.22	0.62
IL-17A	69.98 \pm 9.88	95.03 \pm 6.44	0.07	56.41 \pm 7.46	102.4 \pm 19.01	0.04	38.13 \pm 10.39	45.05 \pm 8.78	0.62
IFN γ	295 \pm 72.78	385.4 \pm 48.6	0.32	357.5 \pm 57.63	593.2 \pm 84.33	0.05	195.4 \pm 65.29	243.3 \pm 104	0.72
IL-6	90.37 \pm 6.45	67.23 \pm 9.79	0.08	126.9 \pm 19.52	69.64 \pm 6.93	0.01	61.24 \pm 18.05	42.28 \pm 12.17	0.44
IL-1 β	91.99 \pm 5.23	58.68 \pm 7.29	0.006	142.4 \pm 28.24	60.35 \pm 4.42	0.01	47.66 \pm 14.08	29.81 \pm 7.66	0.31
TNF α	163.5 \pm 7.16	92.06 \pm 21.04	0.01	194.6 \pm 28.03	100.1 \pm 14.24	0.008	94.77 \pm 14.24	57.45 \pm 14.51	0.13
CCL2	32.51 \pm 1.54	24.1 \pm 5.64	0.19	43.97 \pm 7.25	25.42 \pm 1.37	0.02	28.1 \pm 4.99	19.72 \pm 1.23	0.14
CXCL1	197.6 \pm 8.85	142.5 \pm 20.93	0.04	183.4 \pm 17.75	123.7 \pm 10.5	0.01	105.6 \pm 6.49	77.86 \pm 9.64	0.04
Liver enzymes**									
ALT	142.5 \pm 52.5	0.00 \pm 0.00	0.004	111.7 \pm 70.77***	60.0 \pm 35.0***	0.32	0.00 \pm 0.00	0.00 \pm 0.00	NA
AST	159.6 \pm 39.79	101.0 \pm 1.87	0.18	134.0 \pm 15.28***	97.0 \pm 8.0***	0.06	105.0 \pm 25.45	89.0 \pm 5.1	0.55

*: Samples with not detectable levels were not included in the analysis.

** : levels are U/L.

*** : n=5, 8 months after DEN

Table 2. Serum cytokine and liver enzyme levels in HCC-bearing mice.

Cytokines and ALT-AST were detected in sera of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ HCC-bearing mice at different time-points.

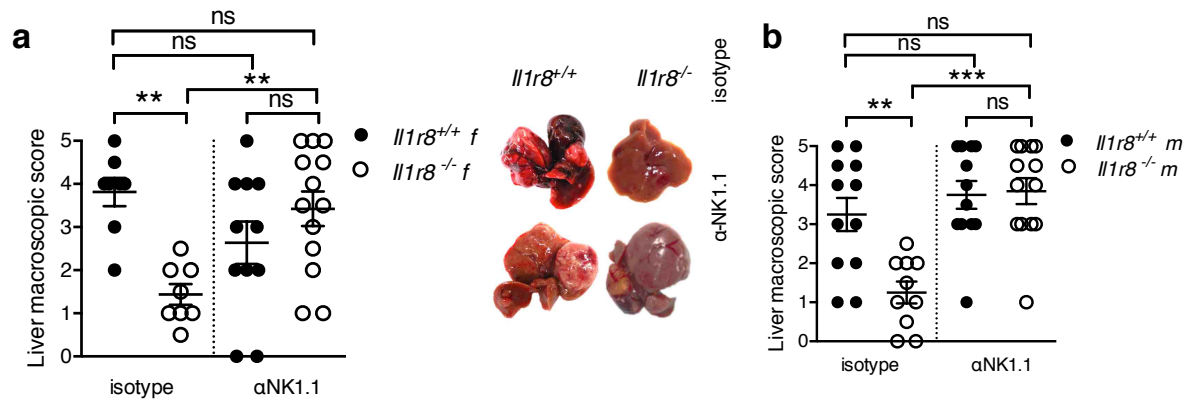


Figure 11. IL-1R8-deficient NK cells are protective against DEN-induced HCC. (a, b) Macroscopic score of liver lesions in female (a) and male (b) $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice upon NK cell depletion. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

5.2.2 IL-1R8 inhibits NK cell anti-metastatic activity

NK cells play a major role in controlling hematogenous metastases and we therefore addressed the role of IL-1R8 in NK cells a model of sarcoma-derived lung metastases and a model of colon cancer-derived liver metastases. The 3-MCA derived fibrosarcoma cell line MN-MCA3 was injected intramuscularly in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice and primary tumor growth and lung-metastasis were analysed. As shown in Figure 12A, the number of lung metastases was significantly lower in $Il1r8^{-/-}$ mice 25 days upon MN-MCA3 injection, whereas we did not observed difference in primary tumor growth between the two groups

(Figure 12B). The frequency of total NK cells and mature CD27^{low} NK cells was higher in *Il1r8*^{-/-} lungs (Figure 12C and 12D). To address the involvement of NK cells in the protection observed in *Il1r8*^{-/-} mice, we depleted NK cells treating mice with a specific anti-NK1.1 antibody *in vivo*. We then performed *in vivo* imaging 3-dimensional analysis through Fluorescence Molecular Tomography (FMT) technology, which is based on 2-deoxyglucose (2-DG) fluorescence. Through this technique it is possible to obtain a precise localization of the tumor and a quantification in deep tissue. 2-DG is a glucose molecule, with the 2-hydroxyl group replaced by hydrogen, in order not to undergo further glycolysis and it selectively marks cells with a high glucose uptake, such as tumor cells. The fluorescent substrate was injected i.v. in the animal 24 hours and the analysis was performed using a trans-illuminator. The signal in the lung showed that the protection was completely abolished in NK cell-depleted *Il1r8*^{-/-} mice. This result was also confirmed by macroscopic count of lung metastases. (Figure 12E, 12F). To address the involvement of IL-18/IL-18R axis in IL-1R8-dependent regulation of NK cells in this model, we neutralized IL-18 and IFN γ in sarcoma-bearing mice, for the entire duration of the experiment. As shown in the new Figure 12G, both IFN γ and IL-18 neutralization abolished or dramatically reduced the phenotype of IL-1R8-deficient mice. On the contrary, IL-1R1/IL-1R8-double deficient mice showed the same phenotype as IL-1R8-deficient mice, indicating that IL-1 signaling did not contribute to the protection. Since IL-1R8 is known to be involved in Th17 cell polarization and it is expressed by both CD4 and CD8 T cells (Figure 1A, 1B, 1F), we depleted IL-17A and CD4/CD8 to assess the role of T cells in IL-1R8-mediated protection. The depletion of T lymphocytes or the effector molecule IL-17A did not impact on the protective role of IL-1R8-deficiency in the control of metastases, even though the depletion of T cells caused an increased number of lung metastases in wild-type mice. Moreover, we did not observe any difference in T cell frequency and T cell subsets (i.e. naïve, effector and memory cells) in IL-

IL18-deficient mice compared to wild-type mice, either in lung or in spleen of tumor-bearing mice (not shown).

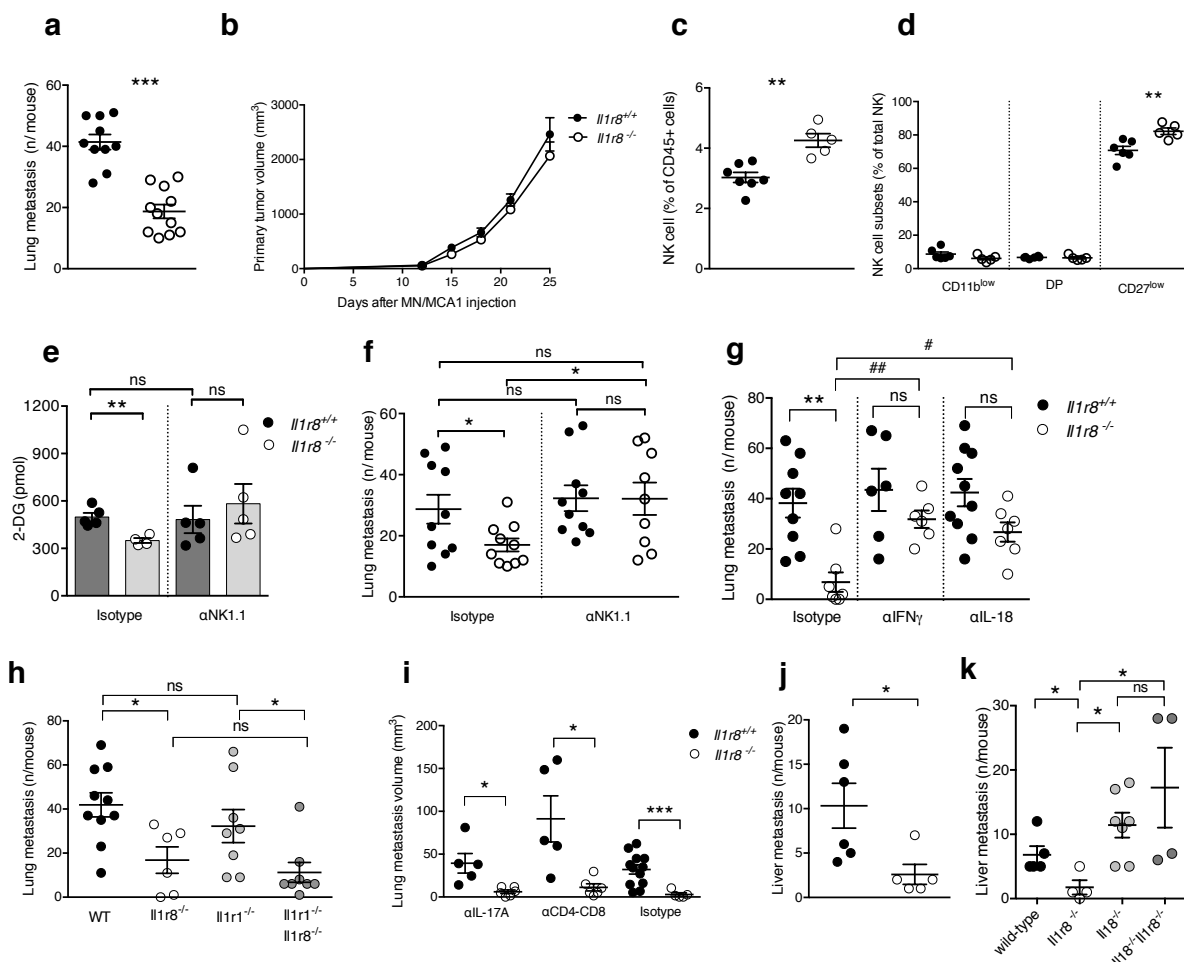


Figure 12. Role of IL-1R8 in NK cell anti-metastatic activity. (a) Number of spontaneous lung metastasis in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice (25 days after i.m. injection of the MN/MCA1 sarcoma cell line). (b) MN/MCA1 primary tumor growth in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice. (c, d) NK cell (c) and NK cell subset (d) frequency in the lungs of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ MN/MCA1 tumor bearing mice. (e, f) 2-DG quantification (e) and number of metastases (f) in lungs of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ tumor bearing mice upon NK cell depletion. (g) Number of lung metastasis in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ MN/MCA1 sarcoma bearing mice upon after IFN γ or IL-18 neutralization. (h) Number of lung metastases in $Il1r8^{+/+}$ and $Il1r8^{-/-}$, $Il1r1^{-/-}$, $Il1r1^{-/-}/Il1r8^{-/-}$ MN/MCA1-bearing mice. (i) Volume of lung metastases in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ MN/MCA1-bearing mice upon depletion of IL-17A or CD4 $^{+}$ /CD8 $^{+}$ cells. (j) Number of liver metastases in $Il1r8^{+/+}$ and $Il1r8^{-/-}$ MC38 colon carcinoma bearing mice. (k) Number of liver metastases in $Il1r8^{+/+}$, $Il1r8^{-/-}$, $Il18^{-/-}$, $Il18^{-/-}/Il1r8^{-/-}$

*/Il1r8^{-/-} MC38 colon carcinoma bearing mice. (*p < 0.05, **p < 0.01, ***p < 0.001, Mean ± SEM is represented)*

Liver metastasis is a major problem in the progression of colorectal cancer and we therefore assessed the potential of Il1r8^{-/-} NK cells to protect against liver metastasis using the MC38 colon carcinoma cell line injected in the spleen. As shown in Figure 12J, Il1r8^{-/-} mice were protected against MC38 colon carcinoma liver metastasis and in addition, IL-18-genetic deficiency abrogated the protection observed in Il1r8^{-/-} mice (Figure 12K), indicating that the IL-1R8-dependent control of MC38-derived liver metastasis occurs through the IL-18/IL-18R axis.

Finally, to assess the primary role of Il1r8^{-/-} NK cells in the cancer protection, adoptive transfer was used. Adoptively transferred NK cells were detectable 3 and 7 days after injection (Figure 13A, 13B), they displayed proliferative capacity (Figure 3C) and were fully functional, in terms of IFN γ production and degranulation after ex vivo stimulation (Figure 3D, 3E). Interestingly, degranulation and IFN γ production were significantly higher in Il1r8^{-/-} NK cells (Figure 3D, 3E). As shown in Figure 13F-H, adoptive transfer of Il1r8^{+/+} NK cells had no effect on lung and liver metastasis. In contrast, adoptive transfer of Il1r8^{-/-} NK cells significantly and dramatically reduced the number and volume of lung and liver metastasis (Figure 13F-H). The anti-metastatic activity of Il1r8^{-/-} NK cells was particularly evident in the liver colorectal metastasis model, with 47 ± 13 secondary lesions in control mice and 5 out of 6 mice free of secondary lesions in mice transferred with Il1r8^{-/-} NK cells (Figure 13H). Given the natural history and clinical challenges of colorectal cancer, this observation has potential translational implications. Thus, IL-1R8 genetic inactivation unleashes NK cell mediated resistance to carcinogenesis in the liver and amplifies the anti-metastatic potential of these cells in liver and lung in a NK cell-autonomous manner.

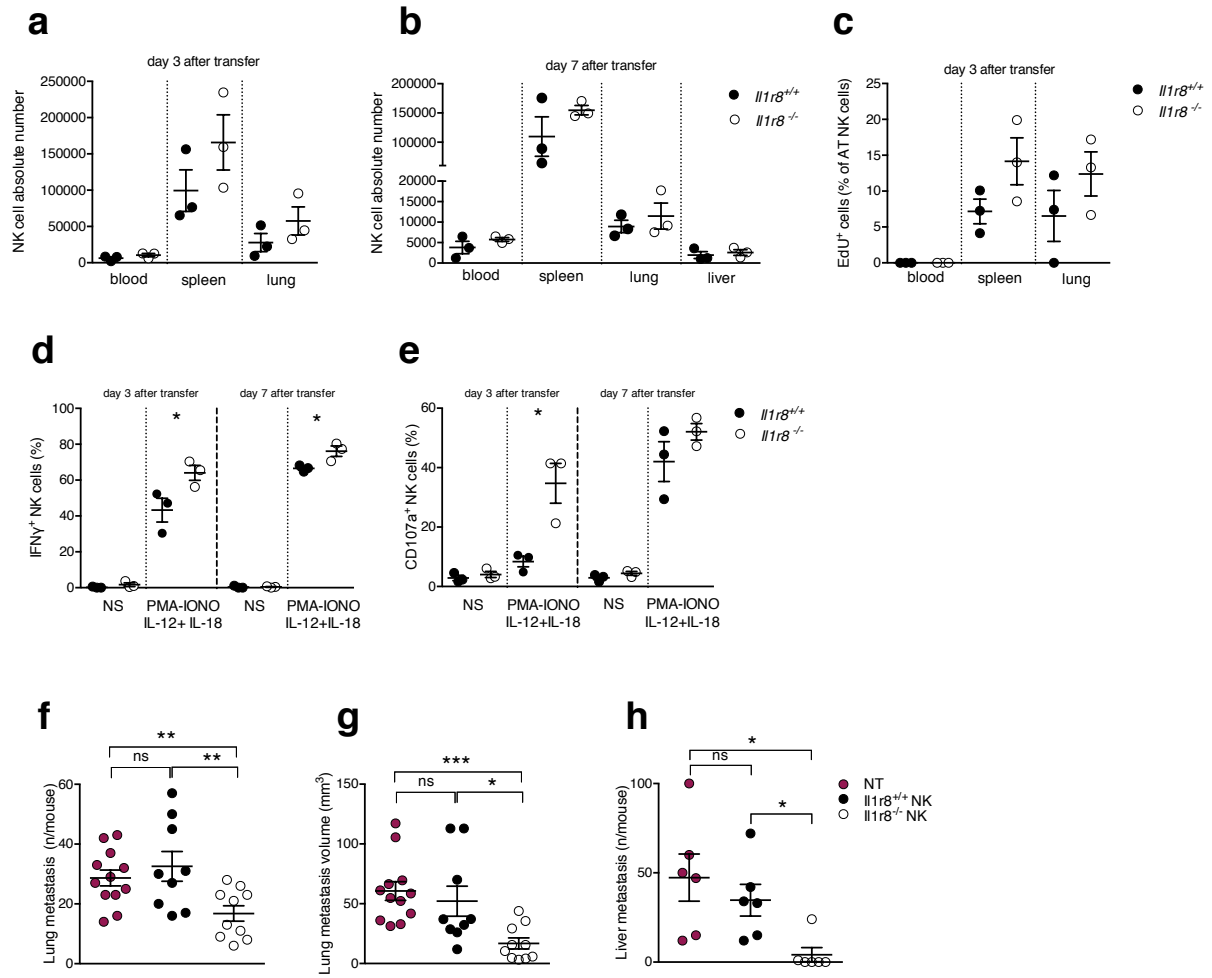


Figure 13. Adoptive transfer of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cells in tumor bearing mice. (a, b) $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cell absolute number three or 7 days after adoptive transfer. (c) In vivo $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cell proliferation three days after adoptive transfer. (d, e) Ex vivo IFN γ production (d) and degranulation (e) upon 4-hour stimulation with PMA-Ionomycin, IL-12 and IL-18 in adoptively transferred $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cells. (f, g) Number (f) and volume (g) of lung metastases of $Il1r8^{+/+}$ MN/MCA1 sarcoma bearing mice after adoptive transfer of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cells. (h) Number of liver metastases of $Il1r8^{+/+}$ MC38 colon carcinoma bearing mice after adoptive transfer of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ NK cells.

(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

5.3 Relevance in viral infection

5.3.1 IL-1R8-deficiency is associated with a more efficient viral control in liver

MCMV infection is a well-characterized model that resembles the systemic and controlled viral infections in immunocompetent individuals. To investigate whether IL-1R8 regulates NK cell effector functions in the context of viral infections, we analyzed MCMV control and NK cell activity in *Il1r8^{+/+}* and *Il1r8^{-/-}* MCMV-infected mice. In particular, we infected mice with wild-type and Δ m157 MCMV, a mutant virus with a deletion of the m157 gene that compromises virus control by Ly49H⁺ NK cells, which recognize the viral-encoded protein m157 and consequently undergo proliferation and activation.

We infected wild-type and IL-1R8-deficient mice intravenously with 5×10^5 PFU of wild-type and Δ m157 MCMV to characterize the viral control in presence or in absence of IL-1R8. Viral load was determined by plaque assay in the homogenates of liver, lung and spleen. As shown in Figure 14A, the liver viral titer was lower in *Il1r8^{-/-}* compared to *Il1r8^{+/+}* mice in both infections and both time-points, indicating that IL-1R8-deficient mice were able to clear the virus more efficiently. In lung and spleen no difference in terms of viral titer was observed.

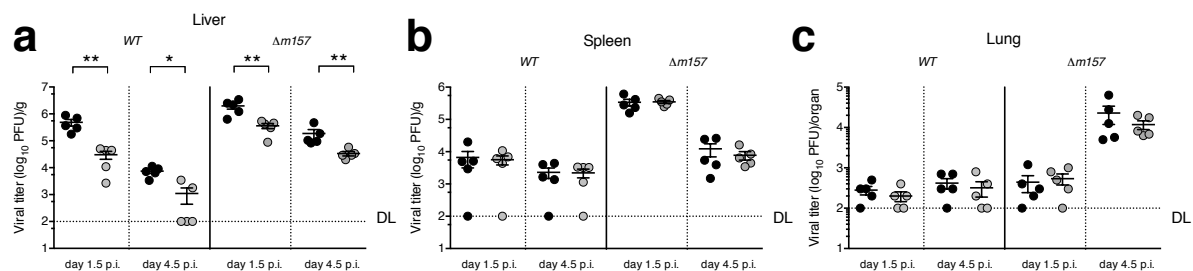


Figure 14. MCMV titer in *Il1r8^{+/+}* and *Il1r8^{-/-}* mice. (a-c) Viral titer assessed by plaque assay in liver

(a), spleen (b) and lung (c), 1.5 and 4.5 days post infection with wild-type and $\Delta m157$ MCMV. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

5.3.2 IL-1R8-deficient NK cells displayed enhanced effector functions after infection

To assess whether NK cells were involved in the antiviral protection, we analyzed NK cell effector functions in liver and spleen upon infection. As shown in Figure 15, NK cells displayed enhanced effector functions in absence of IL-1R8, at day 1.5 post infection. Indeed, in case of infection with wild-type MCMV, 1.5 days post infection correspond to the peak of the innate immune response. At 4.5 days post infection the virus is controlled and indeed NK cell activity decreased. IL-1R8-deficient NK cells produced higher levels of IFN γ in both liver (Figure 15A) and spleen (Figure 15B) and degranulated more in both liver (Figure 15C) and spleen (Figure 15D), at day 1.5 post infection. A more sustained NK cell activation in terms of IFN γ (Figure 15B) and degranulation (Figure 15D) was also observed in case of $\Delta m157$ infection in spleen. No difference in terms of Granzyme B production was observed between Il1r8^{+/+} and Il1r8^{-/-} mice upon infection (Figure 15E, 15F). Perforin production increased in Il1r8^{-/-} mice in spleen (Figure 15H), but not in liver (Figure 15G), at day 1.5 post infection, in both WT and $\Delta m157$ infections. Interestingly, NK cell degranulation (Figure 15C), Granzyme B (Figure 15E) and Perforin (Figure 15G) production was lower in Il1r8^{-/-} mice 4.5 days post infection, in case of WT MCMV. NK cell activation decreases during viral clearance and the reduced NK cell activation observed in Il1r8^{-/-} mice reflected a more efficient viral control in absence of IL-1R8. Moreover, we analyzed cytokine levels in sera of infected mice (Figure 16).

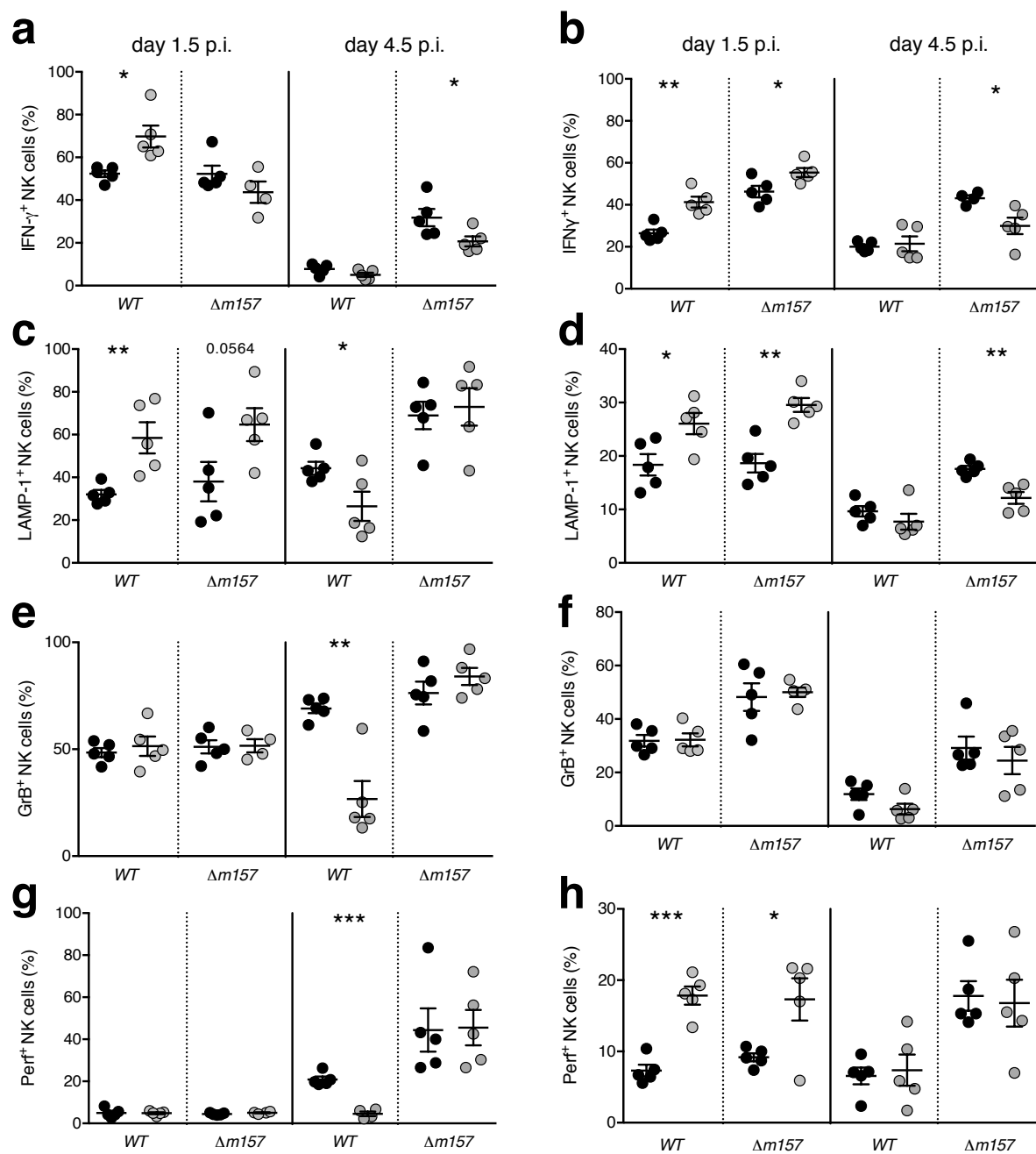


Figure 15. NK cell effector functions in infected mice. (a-h) Frequency of IFN γ ⁺ (a, b), CD107a⁺ (c, d), GranzymeB⁺ (e, f) and Perforin⁺ (g, h) NK cells in liver (a, c, e, g) and spleen (b, d, f, h) of *Il1r8*^{+/+} and *Il1r8*^{-/-} infected mice (1.5 and 4.5 days post infection), with wild-type and $\Delta m157$ MCMV. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

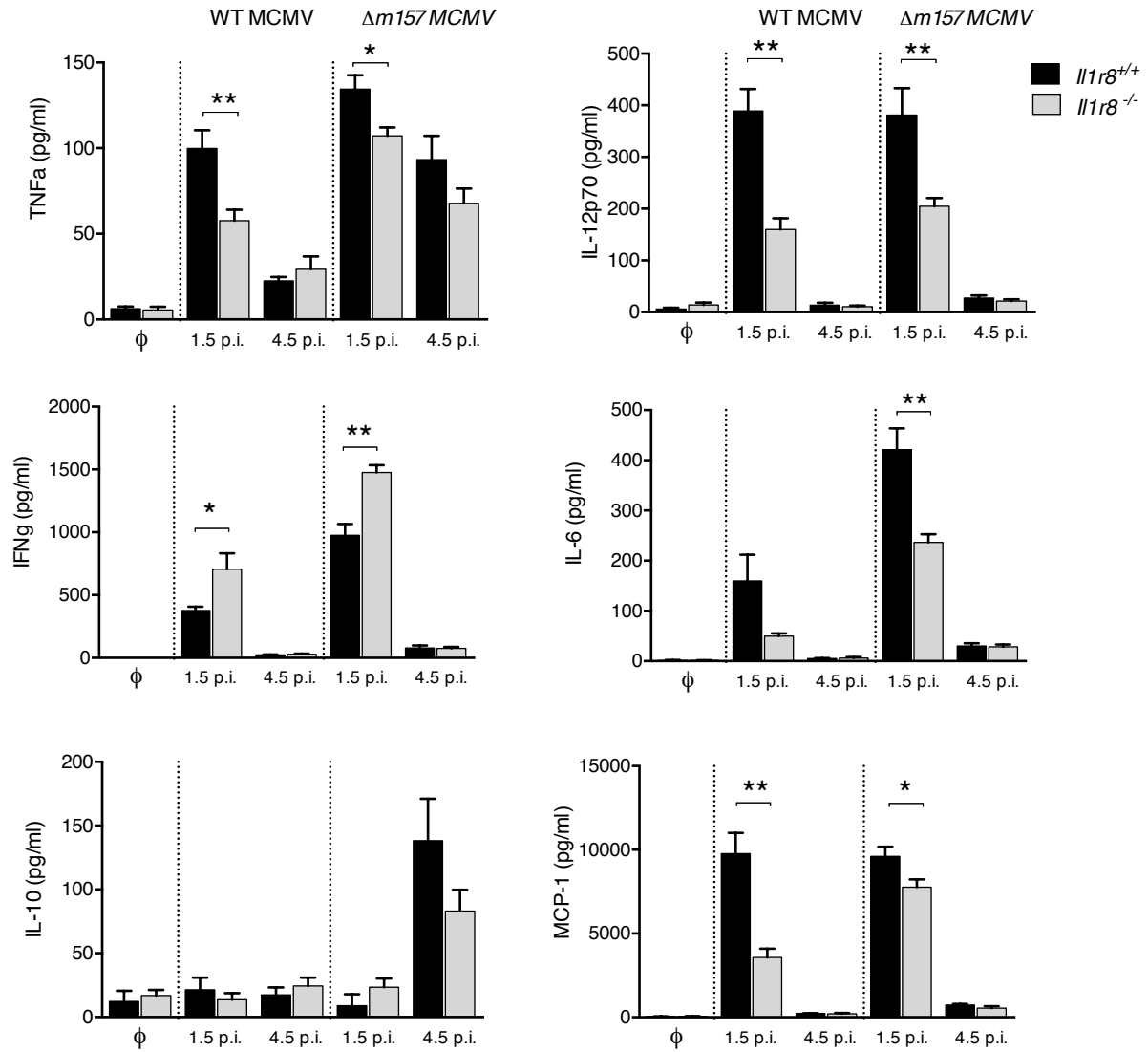


Figure 16. Serum cytokine levels in infected mice. Cytokine serum levels in *Il1r8*^{+/+} and *Il1r8*^{-/-} infected mice (1.5 and 4.5 days post infection), with wild-type and $\Delta m157$ MCMV. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

Levels of pro-inflammatory cytokines (i.e. TNF α , IL-12p70, IL-6, IL-10, CCL2) were reduced in *Il1r8*^{-/-} mice, reflecting the reduced viral titer. Interestingly, IFN γ , which is an important anti-viral mediator produced by cytotoxic lymphocytes including NK cells, was detected at higher levels in sera of IL-1R8-deficient mice (Figure 16). This is consistent with

the more efficient viral control observed in Il1r8^{-/-} mice and the enhanced IFN- γ production observed intracellularly in NK cells in liver and spleen.

Collectively, these results indicated that IL-1R8-deficient mice are protected against MCMV infection and this correlated with increased NK cell activation.

5.3.3 The protection observed in IL-1R8-deficient mice is dependent on NK cells

To demonstrate whether the protection we observed in Il1r8^{-/-} mice was directly dependent on NK cells and not influenced by other cell types expressing IL-1R8, we first analyzed the involvement of T cells in the phenotype, since T cells are crucial mediators of antiviral activities. We observed that the depletion of T cells did not affect the protection in liver (Figure 17A) and IFN γ production was not altered in absence of IL-1R8, in both liver and spleen, upon stimulation with specific viral-encoded peptides (i.e. m38 and m45) (Figure 17B-17E). This indicated that T cells were not involved in the viral attenuation observed in the absence of IL-1R8. However, since CMV is mainly controlled by NK cells, we cannot exclude the role of IL-1R8 in T lymphocytes in viral models where the T cell-mediate response is relevant.

To directly demonstrate that IL-1R8-deficient NK cells were responsible for the viral attenuation, we performed adoptive transfer of sorted NK cells (NK1.1⁺ CD3⁻) in newborn mice, which were infected at day 1 after birth and transferred with NK cells at day 2 post infection. We decided to use newborn mice, instead of adult mice, to avoid the control of MCMV mediated by endogenous NK cells that occurs in adult immune-competent mice. In newborn mice, NK cells are not differentiated yet and the virus is not controlled. As shown in Figure 18, the transfer of IL-1R8-deficient NK cells provided protection in liver, spleen, lung

and brain, demonstrating that IL-1R8 plays a key role in the regulation of NK cell antiviral activities. The protection observed in the brain is particularly important in the context of the congenital infection of CMV, in which the virus infects the developing fetus and can reach the CNS, causing long-term neurodisorders.

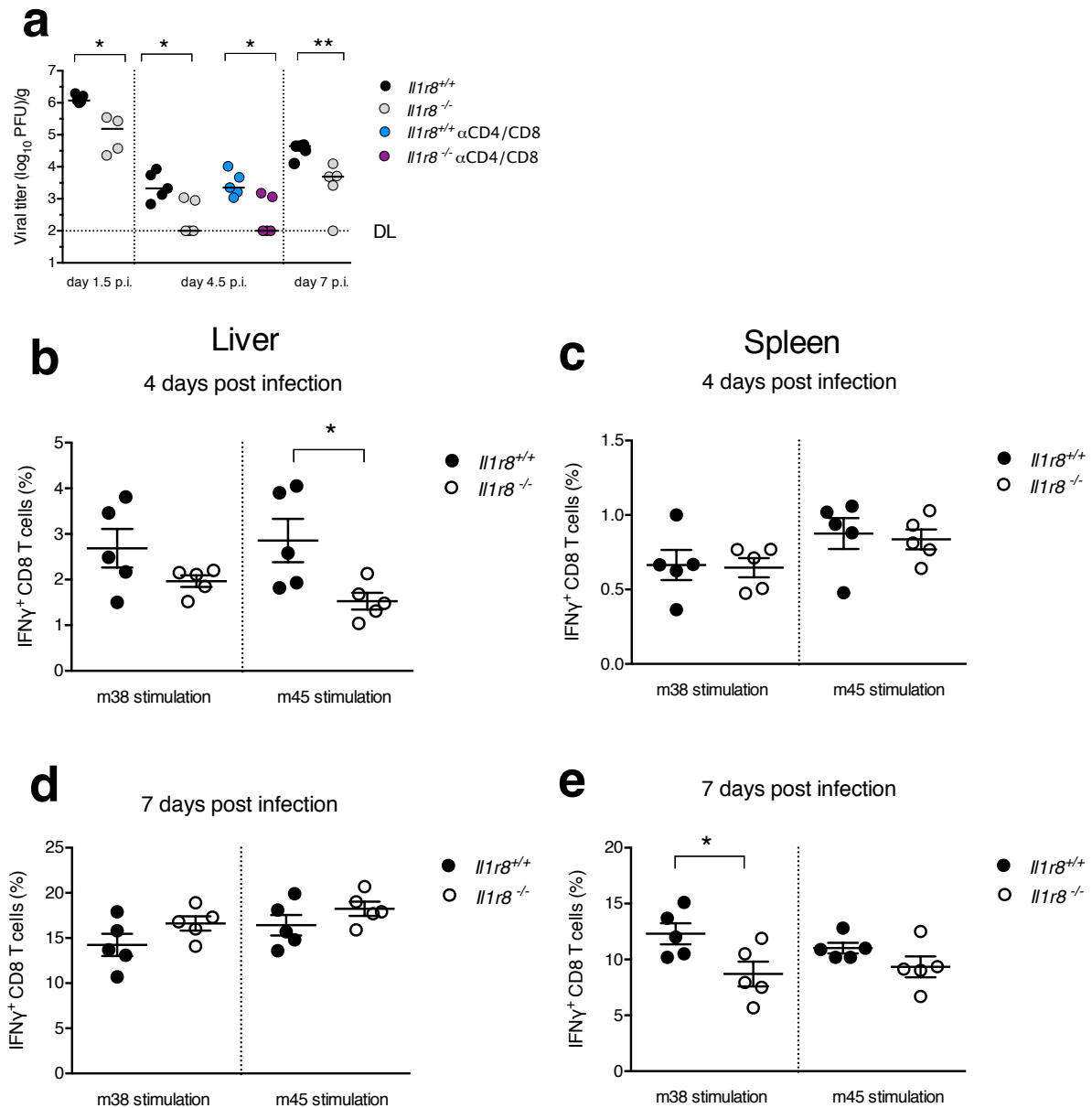


Figure 17. T cell contribution in MCMV infection. (a) Viral titer in liver of *Il1r8*^{+/+} and *Il1r8*^{-/-} infected mice at day 4.5 post infection, upon CD4⁺/CD8⁺ T cell depletion. (b-e) IFN γ production by CD8⁺ T cells 4 (b, c) and 7 days post infection (d, e), in liver (b, d) and spleen (c, e), upon stimulation with m38 and m45 peptides. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mean \pm SEM is represented)

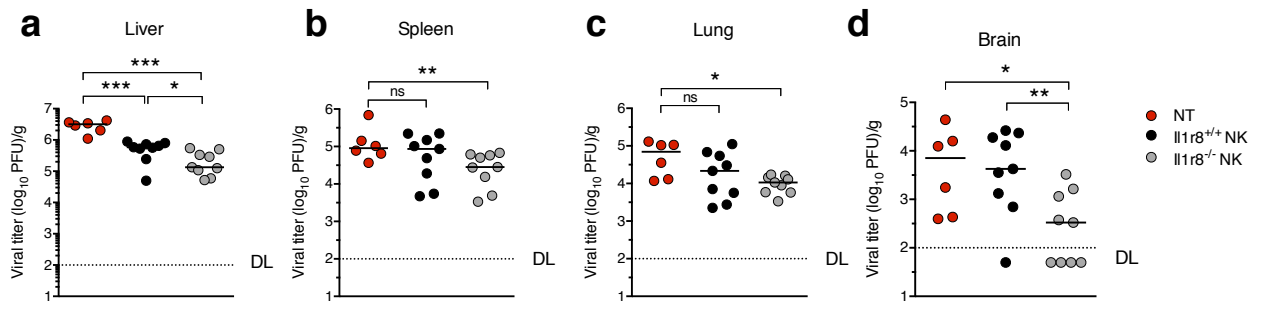


Figure 18. NK cell adoptive transfer in newborn infected mice. (a-d) Viral titer in liver (a), spleen (b), lung (c) and brain (d) of newborn wild type mice upon adoptive transfer of Il1r8^{+/+} and Il1r8^{-/-} NK cells (7 days post infection). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean \pm SEM is represented)

6. Discussion and future perspectives

Unleashing or redirecting the effector functions of NK cells, and in general of cytotoxic lymphocytes, is a promising approach in oncology that has recently led to improved treatments for several types of cancer. It is based on the fundamental concept of readdressing the equilibrium of activating and inhibitory signals, which regulate the immune system in physiological and pathological conditions [215]. In this study, we focus on the role of IL-1R8, a negative regulator of the IL-1 family, in NK cells and we addressed the relevance of IL-1R8 blockade to sustain and enhance NK cell antitumor and antiviral activities.

We showed that human and murine NK cells expressed high levels of IL-1R8 and it was acquired during the maturation process, in line with other inhibitory receptors. Indeed, the expression of negative regulators increases in mature NK cells, which display a lower threshold of activation and need to be finely regulated. Compared to other leukocytes, NK cells expressed higher levels of IL-1R8, mirroring the necessity of a quick and strong inhibition, to avoid an uncontrolled and detrimental response.

IL-1R8-deficient NK cells displayed a more mature phenotype and enhanced effector functions, in terms of IFN γ and GranzymeB production, FasL expression and degranulation, upon stimulation ex-vivo. Moreover, NK cells expressed higher levels of activating receptors (NKG2D, DNAM-1 and Ly49H) at steady-state conditions. This indicated that IL-1R8 mediates a regulatory signal, part of the wide spectrum of inhibitory signals that counterbalance the strong cytotoxic potential of NK cells. In absence of IL-1R8, NK cells are hyper-responsive to inflammatory signals, displaying a lower threshold of activation.

Competitive bone marrow chimeric mice and depletion experiments demonstrated that IL-1R8 directly acts on NK cells, blocking IL-18 signaling. Thus, IL-1R8 acts as an inhibitor of NK cell maturation and effector functions, by tuning IL-18-dependent activation. Bone

marrow precursor analysis and NK cell phenotyping in newborn mice showed that IL-1R8-mediated regulation in NK cells is an early and central event, acting in the first stages of NK cell development, but not affecting NK cell precursors. Indeed, IL-18-driven signaling, which is regulated by IL-1R8, is not considered one of the major modulators of NK cell development and mainly acts in differentiated NK cells [108]. The absence of IL-1R8 determines enhanced and IL-18-driven NK cell maturation, suggesting that an uncontrolled signal of IL-18 in NK cells leads to a mature and hyper-active phenotype.

Other than IL-18, IL-1- and TLR-signaling are regulated by IL-1R8 and could potentially favor NK cell maturation in absence of IL-1R8. Deficient mice demonstrated that IL-1 was not involved in the enhanced NK cell maturation observed in IL-1R8-deficient mice. Since $Il1r8^{-/-}$ NK cells displayed a more mature phenotype at steady-state conditions, we infer that TLR signaling could be driven by commensal-derived agonists. In line with previous reports, flora depletion experiments showed that the microbiota was involved in the stimulation of NK cell maturation, but it was not responsible for the enhanced NK cell maturation observed in absence of IL-1R8. This is possibly due to the fact that instructive signals derived from the microbiota may not have a direct effect on NK cells, as shown by Ganai SC et al. and the role of IL-18 is predominant in IL-1R-deficient NK cells [350].

IL-1R8 therefore emerges as a modulator of NK cell activities and to give relevance to this finding, we investigated the significance of IL-1R8 in the regulation of NK cell effector functions in models of NK cell-mediated immunesurveillance: cancer and viral infections.

NK cells play an important role in the innate immune response against tumor metastasis, whereas the control of solid tumors by NK cells is normally poorly efficient. Nevertheless, NK cell-based immunotherapy approaches that enhance NK cell activation lead to liver carcinoma rejection in the mouse and several clinical trials are ongoing.

In a model of DEN-induced liver carcinogenesis, we observed that IL-1R8-deficiency

determined a reduced tumor development and progression, in terms of number of lesions and disease severity. The depletion of NK cells led to a rescue of the phenotype, indicating that IL-1R8 blocks NK cell antitumoral activity against liver primary tumor. We next analyzed a model of lung metastases and IL-1R8-deficient mice were protected from the development of lung metastasis, derived from a fibrosarcoma cell line (MN-MCA-1), injected in the muscle. The protection was completely abolished upon NK cell depletion. This indicates that, in the absence of IL-1R8, NK cells acquire a stronger anti-metastatic activity and are responsible for the reduced susceptibility of IL-1R8-deficient mice. It is important to note that the depletion of NK cells in wild-type mice did not affect the susceptibility to both lung metastasis and HCC, in line with the hypothesis that NK cell-mediated protection is not sufficient in cancer models and in patients with established tumors. Interestingly, IL-1R8-deficient NK cells acquire the ability to be protective in both models. Given that colorectal cancer-derived liver metastases are the major clinical issue in the treatment of colorectal cancer patients, we addressed the role of IL-1R8 in NK cells in a model of colon cancer-derived liver metastases. In this model, IL-1R8-deficient mice were protected from the development of liver metastases. Importantly, the adoptive transfer of IL-1R8-deficient NK cells provided protection in both lung and liver metastasis models, suggesting a translational potential of this treatment, in the context of NK cell-based immunotherapy.

In a model of viral infection (MCMV), we observed less susceptibility in absence of IL-1R8, associated with increased NK cell effector functions in liver. This indicates that IL-1R8 is responsible for blocking NK cell antiviral activities, as well. The control of MCMV is particularly important in immunocompromised individuals and in case of bone marrow transplantation, the reactivation of the virus is associated with worst outcome [306].

IL-1R8 therefore emerges as a checkpoint of NK cell effector functions and the blockade of this molecule might represent a promising immunotherapeutic approach, aimed at enhancing

NK cell antitumoral and antiviral activity. Importantly, the partial silencing of IL-1R8 in human primary NK cells was associated with enhanced effector functions and IL-1R8 expression level in NK cells was inversely correlated with IFN γ production in healthy donors. This indicates that IL-1R8 activity in NK cells is conserved in humans, giving relevance to IL-1R8 blockade in cancer therapies.

IL-1R8-deficient mice show exacerbated inflammation in response to ILR and TLR signaling, which can promote cancer and since we used IL-1R8 total knock-out mice, the protective effect we observed in the liver and lung might be an underestimation of the potential activity of IL-1R8-deficient NK cells.

It is important to note that NK cell hyper-activation may be detrimental in certain conditions and the regulation of NK cell reactivity exerted by inhibitory molecules is necessary to avoid unspecific and autoimmune responses. Indeed, certain KIR/HLA haplotypes are associated with enhanced NK cell activation and autoreactivity that can lead to autoimmune reactions [328]. Nevertheless, in patients with malignancies the enhanced activation of antitumoral activities represents the major clinical need, even though potential autoimmune reactions need to be taken into account. In this regard, the next step of this investigation will be the characterization of IL-1R8 in human NK cells in pathological conditions to understand its eventual modulation and its role in regulating NK cells in contexts where NK cell effector functions can be hampered, such as hematological malignancies and liver cancer. We will also characterize the correlation of IL-1R8 expression in NK cells with clinical outcome in patients and we will then develop and test blocking reagents for the specific inhibition of the molecule in NK cells. Finally, it will be interesting to characterize the potential role of IL-1R8 in the other subsets of innate lymphoid cells (non NK-ILC1, ILC2 and ILC3) and cytotoxic T cells.

7. Bibliography

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8. Relevant papers